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Prevalence and Antimicrobial Resistance of *Campylobacter jejuni* in Alberta Slaughter and Retail Chicken

by

Mary A. VanderKop

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Medical Sciences – Public Health Sciences

Edmonton, Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Prevalence and Antimicrobial Resistance of Campylobacter jejuni in Alberta Slaughter and Retail Chicken* submitted by Mary A. VanderKop in partial fulfillment of the requirements for the degree of Master of Science in Medical Sciences – Public Health Sciences.

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Abstract

The prevalence of *Campylobacter jejuni*, a contaminant of chicken that causes human gastroenteritis and develops resistance to fluoroquinolone antibiotics used for treatment, was established by cross-sectional survey in spring 2001, in Alberta, Canada. The prevalence in ceca from slaughtered chickens (n=400) was 35%, with 53% prevalence in rinses of retail chickens (n=417). *C. jejuni* isolation was associated with retail brand, which may reflect differences in carcass processing.

From 9 to 13% of both cecal and retail *C. jejuni* isolates were resistant to fluoroquinolone antibiotics, and resistance was not associated with factors related to the source. Isolates of *C. jejuni* from cecal samples required lower minimum inhibitory concentrations of erythromycin than retail chicken isolates. Pulsed-field gel electrophoresis with *Sma*-1 provided weak discrimination between the populations of C. jejuni, and would not be recommended for distinguishing strains. Ongoing monitoring is desirable to establish whether antibiotic resistance persists in *C. jejuni* from poultry.

Dedication

I dedicate this thesis to my husband, Perry VanderKop; without his unwavering support for me and for the work, it would not have been completed. Words cannot express how grateful I am – but I thank you for always being there. This work is for you, with all my love.

I also dedicate this to my parents, Lorne and June Dignean, for giving me their love of learning that made the work a joy because of the challenges. Thank you for everything.

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List of Abbreviations

AAFRD	Alberta Agriculture, Food and Rural Development
CDC	Centers for Disease Control and Prevention
CFIA	Canadian Food Inspection Agency
HR	Hazard ratio
MIC	Minimum inhibitory concentration
NARMS	National Antimicrobial Resistance Monitoring System
OR	Odds ratio
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RR	Relative risk
UK	United Kingdom
USA	United States of America

CHAPTER 1

Introduction

Campylobacter jejuni (*C. jejuni*) is the most frequently confirmed bacterial cause of food borne illness in humans. There were 2 to 4 million cases of enteritis attributed to this organism annually in 1999 in the USA (NARMS: Enteric Bacterial Working Group 2003, Zirnstein *et al* 1999) and 58,000 cases were reported in 2000 in England and Wales (Hartnett *et al* 2001). Case numbers are typically underestimated and accurate numbers for Canada are not available. Enteritis from *C. jejuni* has been linked to consumption of undercooked poultry and contact with raw poultry products (Altekruse 1998, Altekruse *et al* 1998, Bryan and Doyle 1995). Colonization of the intestine by *C. jejuni* does not typically produce clinical signs in poultry species, but the bacteria are abundant in fecal material and have been found frequently on retail chicken (Flynn *et al* 1994, Kotula and Pandya 1995, Powell *et al* 1995, Wassenaar *et al* 1998). Chickens that shed the bacteria are a source of cross contamination during processing, and the bacteria are difficult to eliminate from the processed chicken carcasses without interfering with consumer acceptance of the final product or processing method (Kotula and Pandya 1995).

The prevalence of *C. jejuni* and other *Campylobacter* species is not known for broiler chickens or retail chicken meat in Alberta. There is no current information on the level of resistance of these bacteria to antibiotics that are used in poultry production and those used for treating human infections. The use of antibiotics in livestock has been implicated as a cause of resistance in bacteria that are pathogenic to humans, and this association may limit the availability of veterinary drugs to control disease in livestock. Several prevalence surveys have been performed in Canada, but the number of samples was small and there have been no comparisons between *C. jejuni* isolates from intestinal content of broilers with those obtained from retail chicken. With the growing importance of food safety issues to consumers and the implications of bacterial

resistance to drugs used to treat human infections, the prevalence of *C. jejuni* in Alberta and the resistance of isolates to antibiotics should be documented as a basis for future surveillance. It is worthwhile to investigate possible associations between the source of the chickens and whether *C. jejuni* could be isolated from them.

CHAPTER 2

Research Objectives

There were three primary objectives of this research:

- to estimate the prevalence of *C. jejuni* in cecal content of broiler chickens arriving at federally inspected slaughter plants in Alberta, and establish the level of resistance of these isolates to erythromycin and three fluoroquinolone antibiotics;
- to obtain the same information on whole body washes of broiler chickens available for retail purchase at major urban grocers in Alberta;
- to establish whether the *C. jejuni* isolates obtained from these two populations of chickens could be distinguished by pulsed-field gel electrophoresis (PFGE) patterns or antimicrobial resistance profiles.

Secondary objectives were to determine whether the prevalence of *C. jejuni* in cecal content of slaughter chickens or whole retail chickens was associated with variables related to the source of the isolates.

The null hypotheses to be tested were:

- the isolates of *C. jejuni* from cecal content of broiler chickens do not differ from the isolates of *C. jejuni* from whole body washes of retail chickens with respect to PFGE patterns or resistance to erythromycin and three fluoroquinolone antibiotics
- the frequency with which *C. jejuni* was isolated from cecal content of broiler chickens and the resistance of these isolates to fluoroquinolone antibiotics and erythromycin were not associated with the slaughter facility, the slaughter company, the day of the week the sample was collected nor the city of origin
- the frequency with which *C. jejuni* was isolated from retail chicken and the resistance of these isolates to erythromycin and three fluoroquinolone antibiotics

were not associated with the store company, the chicken brand, the day of week the sample was collected nor the city of origin.

CHAPTER 3

Background

3.1 *Campylobacter* Bacteriology

Campylobacter is a genus of gram-negative, slender curved rod-shaped bacteria with a single polar flagellum. The species, such as *C. jejuni*, that cause human gastrointestinal illness are thermophilic (grow best at 42°C) and micro-aerophilic (grow in the presence of minimal oxygen). They require an enriched medium and protection from drying for optimum growth. Isolates are biotyped according to various systems, which provide methods of tracking for epidemiology (Stanley and Jones 1998). *Campylobacter* is serologically very heterogeneous (Hilton *et al* 1997), but the same serotypes of *Campylobacter* have been isolated from human clinical cases and poultry (Korolik *et al* 1995, Stanley and Jones 1998, Velazquez *et al* 1995a). Pulsed-field gel electrophoresis (PFGE) and polymerase chain reaction (PCR) testing are increasingly relied upon for identifying organisms and for epidemiological tracing of outbreaks (Steele *et al* 1998, Thomas *et al* 1997, Wu *et al* 2002).

C. jejuni is the most prevalent species causing human infection and the most frequently isolated from poultry (Altekruse *et al* 1998, Shane 1992). Although *C. coli* causes similar symptoms in humans, it is more frequently associated with swine than with poultry. *C. lari* is rarely isolated or associated with clinical disease. The species of *Campylobacter* can be distinguished by biochemical tests, by typical antibiotic sensitivity patterns and with PCR testing. Both *C. jejuni* and *C. coli* inhabit the cecum of chickens, where they are embedded in the mucus and use the mucin as an energy source (Mead 1996a). DNA hybridization techniques have detected *Campylobacter* in cecal contents of day old chicks from which the organism could not be isolated (Chuma *et al* 1994) but, in general, it is believed that chickens are not heavily colonized until at least 2 weeks of age. The importance of vertical transmission (from hen to chick

through the egg) has recently been reported (Cox *et al* 2002) but was initially discounted because of the difficulty of culturing the agent from chickens before 2 weeks of age. It has now been confirmed that vertical transmission is possible and may represent a significant mode of contamination (Cox *et al* 2002, Jacobs-Reitsma 1995). Although *Campylobacter* is a fragile organism, the infective dose is very low and as few as 500 to 1,000 organisms can cause clinical illness in humans (Wesley *et al* 1983).

3.2 *Campylobacter* in Poultry

Campylobacter is commonly isolated from poultry. Surveys of poultry carcasses report culture rates of 85 to 90% (Powell *et al* 1995), and 95% of samples (Lee *et al* 1994). In broilers, fecal or cecal surveys have reported *Campylobacter* culture from 88% (James 1998), 56% (Stern *et al* 1995), 82% (Jacobs-Reitsma *et al* 1994a), 80 to 100% (Koenraad *et al* 1996) and 35% of samples (Berndtson *et al* 1996a, Hilton *et al* 1997, Pearson *et al* 1996). Once a flock is colonized, the bacteria spread rapidly between chickens and in most instances the prevalence within a flock will be high (Berndtson *et al* 1996a, Jacobs-Reitsma *et al* 1994a, Pearson *et al* 1996). Within a flock, the number of chickens shedding *Campylobacter* spp. increases with age until slaughter (Achen *et al* 1998, Berndtson *et al* 1996a). The stress of transportation, feed withdrawal or water withdrawal prior to slaughter will also increase the shedding of *Campylobacter* (Byrd *et al* 1998, Mulder 1996, Willis *et al* 1996). The transportation process increases both the number of positive chickens in a flock and bacterial counts on those chickens, presumably from fecal contamination during transport (Stern *et al* 1995). After slaughter of flocks that are colonized with *Campylobacter*, virtually all slaughter equipment is contaminated (Berndtson *et al* 1996a), and chickens with cecal cultures that are negative for the presence of *Campylobacter* can have positive skin cultures after processing (Mead 1996b) suggesting cross-contamination during slaughter and processing. While there have been few studies that trace the level of *Campylobacter* on carcasses through the processing plant (Berrang *et al* 2000), the extent of carcass contamination between slaughter and presentation for retail sale has not been examined.

3.3 *Campylobacter* in Humans

Most cases of gastroenteritis caused by *Campylobacter* in humans are self-limiting. It is responsible for 12% of fatalities from food borne illness (Meer and Misner 1998) and *Campylobacter* is linked with chronic diseases such as Guillain-Barre' Syndrome (Aarestrup *et al* 1997, Altekruse *et al* 1999, McCarthy and Giesecke 2001), as well as Reiter's syndrome and reactive arthritis (Bunning *et al* 1997). It is estimated that only between 5 and 15% of people suffering from food borne illness will seek medical attention (Motarjemi and Kaferstein 1997) and perhaps half of those will actually be documented cases, because most are treated empirically, without confirming an etiology. The estimates of the impact of food borne pathogens are only a fraction of the true societal cost, which includes lost productivity, costs to food production, and control costs. The economic impact of *Campylobacter* in the United States has been calculated to range from $1.2 to 6.6 billion (1995 U.S. dollars), based on estimates of 1.1 to 7 million cases per year (Buzby and Roberts 1997). The economic impact of *Campylobacter* has not been examined in Canada, but one Canadian study referenced in the citation above (Buzby and Roberts 1997) estimated the total number of food borne illnesses at 2.2 million annually, with a cost of $1.3 billion (1985 CDN$). Based on comparison of population numbers, *Campylobacter* could represent between 10 to 20% of that total cost if the situation parallels that in the United States.

3.4 Poultry Production in Alberta

The poultry industry in Alberta operates on a quota system, which had approximately 325 registered broiler chicken producers in 2001. There is some variation in number because farms are bought and sold during the year. Every commercial broiler chicken producer must be registered with the Alberta Chicken Producers organization and the quota that the producer owns determined the number of chickens that they are permitted, and required, to produce and sell annually. Typical Alberta broiler chicken farms have an average of 15,000 to 20,000 (range 5,000 to 200,000) chickens, in barns that each house from 7,000 to 10,000 chickens. Chickens are kept as a large, single

group in each barn, on straw bedding on concrete or earthen floors. Farms have from 1 to 20 barns on the property. Typically, a complete pellet or crumble ration is formulated by feed mills or on the farms and provided to chickens *ad libitum* by automated feed troughs. They receive water through automated drinking systems that carry water in closed lines and deliver it via pressure-operated valves or open bell-shaped water bowls.

Registered hatcheries in Alberta supply one-day-old broiler chicks to the farms and may fill some or all of the barns on a farm at one time. The farms operate on an 8-week cycle, so that chickens are fed for 37 to 43 days and then all of the chickens from all barns at that age are trucked to the slaughter facility. Barns are vacant for about 2 weeks to allow for cleaning before the next group of chicks is brought in. The bedding is typically completely removed between flocks, but the degree to which floors, equipment and walls are washed varies considerably among farms. A small number of chickens is marketed at 5 weeks as Rock-Cornish game hens and a few are raised until 8 to 9 weeks of age and marketed as roaster chickens. There are approximately 6 million broiler chickens slaughtered every 8 weeks in Alberta at four federally inspected slaughter facilities owned by two companies. Most of the chickens produced in Alberta are consumed in province, although the export market is expanding, especially for further-processed products.

CHAPTER 4

Review of Relevant Literature

4.1 Risk Factors for Human Campylobacteriosis

Consumption of poultry products is one of the major risk factors associated with human campylobacteriosis. Other instances of illness have been associated with exposure to puppies or kittens or with consumption of raw milk (Altekruse *et al* 1999, Finch and Blake 1985, Skirrow 1991).

A case-control study (Harris *et al* 1986) examined 218 cases of *C. jejuni* enteritis from Washington State and 526 control cases in 1983 used a questionnaire to compare consumption of various meat products. Consumption of chicken (RR = 2.4) and Cornish game hen (broiler chickens slaughtered at 5 weeks instead of 6 weeks of age) (RR = 3.3) markedly increased the risk of *C. jejuni* diarrhea. Consumption of raw or rare chicken was even more strongly associated (RR = 7.6) and the authors suggest that chicken consumption or cross-contamination may contribute to half the *C. jejuni* clinical cases.

A matched case-control study in Colorado in 1981 (Hopkins *et al* 1984) implicated consumption of untreated drinking water (OR = 10.7), raw milk (OR = 6.9), under-cooked chicken (OR = 2.77) and close contact with cats (OR = 3.2) in laboratory confirmed cases of campylobacteriosis. The association with undercooked chicken was also reported in an investigation of an outbreak Colorado in 1982 involving 11 individuals with enteritis after a barbeque (Istre *et al* 1984). Fecal cultures were positive for *C. jejuni* from two individuals and eight of them had rising serological titres to *C. jejuni*, confirming the diagnosis. The investigation revealed an association between illness and consumption of chicken that was not adequately cooked.

The association with chicken was also substantiated in a case-control study of 45 sporadic cases of campylobacteriosis and age, gender and residence-matched controls at the University of Georgia (Deming *et al* 1987). Students were confirmed as cases if they had *C. jejuni* isolated from fecal samples. Consumption of either chicken (OR = 7.2) or undercooked chicken (OR = 49) in the week preceding illness was identified as a significant risk factor. Again, contact with cats or kittens was also associated with increased risk.

An outbreak of *C. jejuni* enteritis in staff and customers of a catering establishment in England was traced through chicken consumption to the farm of origin by serotyping, which confirmed poultry as the origin in this case (Pearson *et al* 2000). *C. jejuni* was identified in 89% of chickens from one chicken wholesaler and 98% of these were of the serotype identified in the outbreak. The serotype was comparatively rare in samples from a second wholesaler.

In contrast to this, another case-control study of 229 cases in Great Britain found no statistically significant association between campylobacteriosis and consumption of chicken other than at restaurants (Rodrigues *et al* 2001). Travel abroad was the most important risk factor identified and consumption of several foods was found to be protective, including boiled rice, pulses and fruits. There were also no relationships between Campylobacteriosis and household pets, animal contact or delays between purchase of food and refrigeration. This survey examined sporadic cases and indicated some differences between those and small outbreaks that were explored in other studies.

Current evidence suggests that campylobacteriosis in humans is associated with consumption of chicken or raw food contaminated by chicken. A better understanding of the extent to which raw chicken is colonized by *Campylobacter* spp. and the characteristics of the bacteria involved are required to improve food safety controls.

4.2 Prevalence of *Campylobacter* in Slaughter and Retail Chickens

Two poultry processing plants in the Netherlands were surveyed to compare isolation rates of *Campylobacter* from various chicken parts and internal organs, as well as environmental samples and worker's hands (Oosterom *et al* 1983). Almost all chicken cecal samples (23 of 25) were positive for *C. jejuni* and high numbers were isolated. The authors did not perform any statistical analysis to evaluate relationships, but did report that spin chilling of carcasses reduced bacterial numbers markedly, most likely due to air-drying. *C. jejuni* was also isolated from 36 of 40 of the environmental samples including chill water and 19 of 24 samples from worker's hands, indicating that it is widespread in the processing environment.

In 1984, a study of prevalence in Canada on 10 chickens each from 60 flocks at slaughter found 258 of 280 colonic swabs from 28 of 60 flocks positive for *C. jejuni* (Prescott and Gellner 1984a). Though the prevalence of flocks that were positive for *Campylobacter* was below 50%, most of the swabs from individual chickens were positive, indicating that virtually all chickens sampled from the positive flocks were shedding *C. jejuni*.

Campylobacter in Canadian poultry from federally inspected abattoirs was monitored from 1983 to 1986 (Lammerding *et al* 1988). Sampling was random and based on slaughter volume, but distribution of plants was not reported. Five chickens per flock (from the final chill tank) were cultured by whole body rinse and 38.2% of carcasses were positive for *Campylobacter*. Of the 116 *Campylobacter* that were biotyped, 109 were *C. jejuni* and the rest were *C. coli*. Approximately half the lots (a lot was not defined) sampled each year were positive for *Campylobacter*, and from 63.3 to 81.2% of chickens in a positive lot were positive. The frequency of positive lots remained consistent over the three years and this study provides a baseline for overall prevalence of *Campylobacter* in broiler chickens in Canada.

In 1995, in Manitoba, broilers that had failed inspection and were re-processed (trimmed and washed) were compared with those that had passed inspection to determine whether they differed in bacterial contamination (Powell *et al* 1995). *Campylobacter* (80% *C. jejuni*) were detected in whole body and tail washes of 84% of the chickens that passed inspection and 74% of those that were re-processed. These values were not significantly different, and indicated a high level of overall contamination, as well as failure of carcass washing to appreciably reduce *Campylobacter* levels, although the number of coliform bacteria decreased markedly.

Prevalence estimates from England (Jorgensen *et al* 2002) based on whole carcass wash, neck skin and a combination of these methods revealed *Campylobacter* could be cultured from 83% of chicken carcasses, and 98% of these isolates were *C. jejuni*. Only 1.8% of the *C. jejuni* were resistant to erythromycin, but 12 to 13% were resistant to ciprofloxacin. The method of MIC determination and the criteria for categorizing isolates as sensitive or resistant were not detailed. It was interesting that the external packaging was positive for both *Campylobacter* and *Salmonella* spp. and could contaminate other raw food products.

Retail products were examined in Ireland (Moore *et al* 2002) and while 94% of fresh whole chickens and 77% of previously frozen chickens were positive for *Campylobacter* (69% of these were *C. jejuni*) there were no *Campylobacter* isolated from 1,061 ready to eat, fully cooked poultry products. Another survey of Irish retail meats (Cloak *et al* 2001) revealed 65% of 60 samples of homogenized fresh chicken positive for *Campylobacter* but *Campylobacter* could not be isolated from any of the 20 samples of frozen chicken. The prevalence of *C. jejuni* was not reported for poultry samples, but *C. jejuni* accounted for 80% of all *Campylobacter* isolated from retail meat in this survey.

Different sites on the carcass have been compared for detection and total number of *Campylobacter* organisms through processing (Berrang *et al* 2000). Cecal and colon samples had the highest level of *Campylobacter* recovery, but feathers and skin were also heavily contaminated and *Campylobacter* was isolated from 100% of crop contents, but with a lower total number of organisms. This is attributable to feed withholding which is done for 12 hours prior to slaughter to reduce the likelihood of intestinal contamination of the carcass during slaughter. The chickens will consume bedding containing fecal material and the resulting colonization of the crop can result in spread of the organism if the crops are ruptured during the slaughter process. *Campylobacter* contamination did not correlate with other potential indicator organisms such as *Escherichia coli*.

Campylobacter spp. occur with high frequency in multiple tissues of chickens and are a potential source of human infection. There is no recent information on prevalence in Canada, and specifically in Alberta, that might serve as a foundation to estimate the risk of campylobacteriosis in humans.

4.3 Prevalence and Risk Factors for *Campylobacter* in Poultry Production

Epidemiological studies have been conducted since the early 1980's in many countries to estimate prevalence and identify potential risk factors for *Campylobacter* colonization in broiler chickens. A better understanding of risk factors during broiler production could result in intervention strategies to reduce colonization during life and decrease fecal shedding when chickens are slaughtered. This should reduce contamination on chicken carcasses and on retail chicken meat, decreasing the risk to human health.

Five commercial flocks in Israel were monitored for *Campylobacter* by direct and enrichment culture of 24 cecal content samples and cloacal swab cultures at 1, 4 and 8 weeks of age (Pokamunski 1986). Only one flock was positive at 1 week of age, but

three were positive by 4 weeks, with 60% of samples positive for *Campylobacter* (84.2% *C. jejuni*). None of 50 culled and dead-in-shell chicks that were also sampled was positive. No flock descriptive parameters were reported, nor were the selection criteria for flocks or chickens. A convenience sample of chickens would be biased toward those that are easy to catch, which may be associated with leg weakness. Leg weakness is a frequent condition in broilers and affected individuals are more likely to consume litter, so have greater exposure to fecal contamination that could bias the prevalence estimate upwards. Ten water, feed and litter samples were obtained from each barn when chickens were sampled and all were negative for *Campylobacter*, except at four weeks when two litter samples from the flocks that were positive for *Campylobacter* also tested positive. Eleven additional flocks were sampled at 8 weeks and 12 of the total 16 flocks were positive, with 82% of the total number of chickens positive. The sampling method was not specified but the clustering inherent in how chickens are raised and transported would bias the prevalence estimate, though the impact of this cannot be estimated. The authors confirmed 46 different serotypes, with up to 15 different serotypes from one flock, and often several serotypes from the same chicken. An interesting finding was that two flocks, raised concurrently in different barns on the same farm, under identical growing conditions and management, had *Campylobacter* present in 0% and 100% of samples collected at slaughter. This suggests that barriers to colonization can be present on the farm, although they were not investigated in this study.

In Sweden, cecal swabs from broilers from 8 of 16 flocks at slaughter age were positive for *Campylobacter* from cecal swab cultures (enrichment culture) (Engvall *et al* 1986). A total of 200 chickens (pooled to 100 cultures) produced only 2% positive pools from 2 flocks but 83 to 100% positive pools from the remaining six flocks. Descriptive information on the flocks was not reported but 14 flock owners did complete a risk factor questionnaire. The survey results were summarized but were not statistically evaluated and served as a source of speculation that *Campylobacter* infection caused the

marginally higher mortality rate in infected flocks of chickens and that untreated lake water might be a source of the bacteria.

This study also reported observation of four barns on one farm through one production cycle of 40,000 chickens per flock as well as one follow-up flock. Cloacal swabs (pooled in groups of two) were cultured from 10 to 15 chickens per flock at 1, 7, and 14 days of age and all were negative for *Campylobacter*. If this had been a random sample, a sample size calculation reveals that the detectable prevalence would have been 18 to 25% (with 95% confidence), so the negative culture results are only weak support that these flocks were free of *Campylobacter* or that it cannot be isolated from broilers less than two weeks of age. Environmental samples (feed, water supply, litter and droppings) taken sporadically during this trial detected *Campylobacter* in one barn by the third week.

An English study of prevalence in ceca collected at the time of slaughter revealed 37 of 49 broiler flocks on 23 farms were positive for *C. jejuni*, with an average of 50% (range 10 to 75%) within flock prevalence (Humphrey *et al* 1993). These prevalence estimates may be low because samples were stored for up to 4 days at 4°C and some samples were stored for up to 7 days at –20°C, which could reduce the viability of *Campylobacter*. Flock colonization was examined in six flocks on three farms where litter, feed and culled chicks were cultured every 2 to 3 days beginning when the flock was placed in the barn until it became positive for *Campylobacter*. All six flocks were negative until at least 5 days of age, and *Campylobacter* was first detected in chickens by three weeks of age. Feed samples were negative, as was hatchery residue (egg shells and chick fluff) from the breeder flocks that were the source of the chicks placed in these flocks. Isolation results were subjectively compared with farm records, revealing no relationship to water source, drinker type, floor type, house surroundings, flock size, other farming activities and farm hygiene. Three intervention trials between barns on one farm where workers dipped their boots in a 1% phenolic solution before entering

barns delayed *Campylobacter* colonization in the flock from 7 to 10 days to 2 to 3 weeks. In one instance, the chickens on a farm receiving the intervention remained negative until shipment for slaughter. This indicated that biosecurity measures on the farm could prevent transmission of *Campylobacter* into poultry barns.

In Norway, on-farm risk factors for *Campylobacter* were assessed in a randomly chosen flock on each of 176 broiler farms over one year (Kapperud *et al* 1993). Cecal swabs from 26 to 28 chickens from different crates on arrival for slaughter were cultured by direct and enrichment methods. In a double-blinded research study, farmers were interviewed on husbandry, animal contact, flock specific data and production parameters of the flock. Water samples were obtained from 147 farms. *Campylobacter* were isolated from 32 of 176 flocks examined (100% of chickens positive in eight flocks, under 35% positive in 19 flocks). Direct culture was more sensitive than enrichment procedures and successfully identified all positive chickens, while the enrichment method failed to isolate *Campylobacter* from eight flocks that were positive by direct culture. The water source and quality were the most important risk factors identified in this study. Water that had not been disinfected was significantly associated with *Campylobacter* culture from a flock (OR = 3.24) and that from an open surface source was even more strongly associated (OR = 5.24) (Kapperud *et al* 1993). Water quality, determined by total and fecal coliforms was strongly associated with *Campylobacter* culture from a flock (OR = 4.5 and OR = 4.78, respectively), as was *Campylobacter* isolation from water (OR = 12.89) although this may have been a result of fecal shedding rather than the source of flock colonization. Other risk factors identified included tending other poultry before entering the barn, tending swine before entering the barn, autumn vs. other seasons and location by county, but for most variables, the number of flocks with exposure was too low to detect a statistically significant difference. All flocks had good hygienic standards and the low stocking density likely contributed to the low prevalence of *Campylobacter* and low level of most risk factors.

A Swedish research group reported cecal culture of 40 chickens (10 pools of 4) from each of 287 flocks on 18 farms (10% convenience sample) over 1 year (Berndtson *et al* 1996a). *Campylobacter* was detected in 27% of flocks, but false negatives are probable because samples were mailed to the laboratory for culture and the associated delay would permit competitive overgrowth. Risk factors were assessed with a 150-question survey instrument and only univariate analysis was performed so confounding was not adjusted for, nor was nesting of flocks, barn compartments, barns or farms accounted for. The impact of false negative farms on risk factor analysis should also have been considered. The risk of *Campylobacter* colonization increased with increasing flock size, with chicken age at slaughter (especially over 42 days) and with poor biosecurity. The increasing flock size was confounded by more input to the flock (i.e. more food, water and bedding, thus, more potential exposure), higher frequency of mice, more frequently staff who worked with or had recently loaded other poultry, and more likely to have the flocks divided for slaughter. The highest odds ratios were for increased contact with people who had been in other poultry barns, especially flocks that remained for late slaughter after being exposed to catching crews that had visited other farms before removing part of the flock for early slaughter. This confounded the influence of large flock size and late slaughter, but the relationships between these were not explored further. This information tends to support horizontal transmission between flocks by humans acting as vectors.

In the Netherlands (Giessen *et al* 1996) 20 broiler farms were sampled with 10 pools of 10 fresh cecal droppings (that can be visually distinguished from normal feces) over two cycles in two years. Management information was collected, but method of farm selection and whether the management information was representative were not reported. Only direct culture was used, and a cycle was categorized as *Campylobacter* positive if any culture was positive. There was no correlation between cycles on a farm suggesting that 2 weeks for cleaning and down time was sufficient to prevent

transmission from a previous flock to the next one. Increased risk was associated with the presence of other species on the farm (no greater risk with poultry than other livestock) and the use of ground water instead of tap water for cleaning poultry houses. There was a reduced risk of *Campylobacter* with the reported use of detergent to clean barns, cleaning the farm yard between flocks, using boots assigned to each barn while working in that barn, and hand washing before working with the flocks. The findings support that lateral transmission is the important route, although the significance of ground water appears in this study as well, and this requires further exploration, especially relative to broiler production in southern Alberta where irrigation ponds are the major source of water and are open to contamination from wildlife sources.

An extensive longitudinal survey of *Campylobacter* colonization of broiler chickens in Great Britain (Evans and Sayers 2000) followed 100 flocks on 20 sites (50% of broiler production in the UK) over one production cycle between 3 to 4 weeks of age until the birds were positive for *Campylobacter* or were slaughtered. Cloacal and environmental swabs were cultured at a national laboratory and revealed a prevalence at slaughter of 81.6% in broilers, which ranged from 50% positive in flocks slaughtered at approximately 30 days, to 97% positive in those slaughtered at over 50 days. Rapid spread within the flock was confirmed because virtually all samples were positive within a week of the first detection of *Campylobacter*. Numerous exposure variables were assessed and incorporated into a mulitvariate model which revealed increased hazard of colonization (Hazard Ratio HR) associated with broiler houses needing repair (HR = 2.45) and using boot dips only after chicks were placed rather than routinely (HR = 1.58). Removal of dead birds from the barn (HR = 0.64), cleaning and disinfection of the water tank (HR = 0.52), use of boot dips after barn disinfection (HR = 0.47) and weekly changing of boot dips (HR = 0.42) were protective against *Campylobacter* colonization. *Campylobacter* were not isolated from the environment after barn cleaning was completed, and there did not appear to be transmission to subsequent flocks, although the power to draw this conclusion was limited.

Risk factors were similarly examined in 75 broiler farms (60% of national production) in France (Refregier-Petton *et al* 2001) and revealed a flock prevalence of 40.0% *C. jejuni* based on pools of fecal samples collected between August 1999 and May 2000. The flocks were sampled at an average of 35 days of age, culture was by both direct and enrichment procedures with confirmation by PCR and a flock was considered positive if a single sample was positive. Fifty potential risk factors relating to production processes were evaluated in a logistic regression model and seven had a significant impact. Static ventilation was most strongly associated (OR = 20.8 relative to dynamic ventilation) as was more than two poultry houses on the farm (OR = 13.2 compared with two or fewer) followed by a seasonal effect (summer and autumn OR = 6.4 relative to winter, with spring OR = 0.2 relative to winter). Acidification of drinking water used to prevent *Salmonella* infection, more than one stockperson, litter beetles present also increased the odds significantly, and treatment with curative levels of antibiotics during the cycle reduced the odds of a flock being *Campylobacter* positive. None of the measures that reflected sanitation had an impact on risk, but the association with multiple barns and multiple stockpersons suggested that risk of colonization was higher due to spread from surrounding livestock or environment. There was sufficient variability in the risk factors evaluated to have detected an impact if one was present.

Campylobacter is widespread and of diverse serotypes in chickens under commercial production systems from countries around the world. While considerable research has been done on risk factors and methods to reduce the likelihood of colonization on the farm, there are no reliable conclusions. A better understanding of the characteristics of the *Campylobacter* populations that are present in the intestinal tract of birds, compared with those found on retail chicken may help to clarify whether farm risk factors or interventions are important in reducing the risk of human campylobacteriosis.

4.4 Transmission of *Campylobacter* in Poultry

An Australian survey of six *Campylobacter* infected broiler breeder flocks (Shanker *et al* 1986) demonstrated 70 to 82% prevalence in hens (direct culture of cloacal swabs). Fourteen broiler chicken flocks on six farms that originated from these breeder flocks had negative cultures for *Campylobacter* (10 chickens per flock per week) until slaughter at six weeks, indicating that vertical transmission was unlikely. Fertile eggs from the infected breeder flocks were cultured by infusion with selective media, to determine if *Campylobacter* had penetrated to the interior of the egg. *Campylobacter* was recovered from only two eggs of 187 sampled. Both were eggs that were collected from the floor, rather than from nests, which may have increased fecal contamination of the shell or damage to shell integrity and favored penetration by the *Campylobacter*. In addition, *Campylobacter* was not detected in two samples (142 and 193 fertile eggs) from a hatchery, representing unspecified breeder flocks. Experimental infection of 257 fertilized eggs by shell swabbing, vacuum treatment or immersion was unsuccessful. The hatch rate of 162/257 for exposed eggs exceeded that for the control eggs, and *Campylobacter* could not be isolated from any of the chicks or any of the unhatched eggs. When *Campylobacter* was injected directly into the albumin of 167 hatching eggs there was a significant ($p < 0.001$) decline in hatchability, to 7% from 56% (saline-injected controls) and *Campylobacter* was isolated from two of the 12 chicks that hatched as well as all the inoculated unhatched eggs. This study suggested that vertical transmission of *Campylobacter* was unlikely in typical production conditions. Colonization of the chick could only be introduced by injection into albumin, and there was severe depression of hatchability that would effectively prevent transmission to broiler flocks under production conditions.

A Japanese study (Chuma *et al* 1994) reported detection of *C. jejuni* in day old broiler chickens by DNA hybridization methods. These methods did not detect viable organisms, and no *C. jejuni* were isolated by traditional culture, but this raised the possibility of bacterial invasion in the hatchery or during transport to the farms. Egg

transmission of a non-viable form of the bacteria was also a possible explanation for this finding.

Transmission of *Campylobacter* through fertile eggs has now been reported in the United States of America (USA) and the relationship between *Campylobacter* positive breeder flocks and broiler progeny has been examined (Cox *et al* 2002). All of 14 breeder flocks sampled (25 fecal cultures per flock) were positive for *Campylobacter*, despite very high biosecurity in these flocks. All but three of 35 commercial broiler flocks had *Campylobacter* isolated, with 8 to 100% of samples positive. The 6-week-old broilers were more heavily colonized than breeder flocks (age not reported). The three broiler flocks that were known to be progeny of an infected breeder flock had very high levels of shedding (42, 82 and 100% positive), but statistical differences were not reported. The finding of *Campylobacter* shedding in mature breeder chickens suggests that there is continual re-colonization or a lack of immunity and clearing of the organism with intermittent shedding through life, both of which represent a concern if egg transmission is a factor.

Many questions remain with respect to the means by which *Campylobacter* is transmitted between chickens. Transmission appears to occur rapidly among chickens within an exposed flock but potential role of transmission from the hen to offspring through the egg complicates how control measures might be implemented on the farm to reduce *Campylobacter* shedding by broilers at slaughter.

4.5 Bacteriology and Molecular Biology Methods

Campylobacter require elevated temperature and reduced oxygen levels to grow and they are sensitive to extremes of environment. The infective dose is low, so it is important to be able to detect low numbers on contaminated tissues, especially meat that might be meant for human consumption. Various methods, including culture of homogenized tissue, fecal material and cecal content have been used to enumerate these

bacteria. In general, less destructive methods that can be applied at various stages in the production process would be preferred.

Whole body wash is increasingly recognized as the most effective means of culturing poultry for organisms of enteric origin (Russell *et al* 1997). The method can detect very low numbers of contaminants with an uneven distribution over the carcass. Sterile water in a plastic bag large enough to allow vigorous agitation is required. The method can be physically tiring, but automated shakers can be used. A significant advantage is that the method is non-destructive and the carcass is not interfered with, so remains suitable for use and sale after sampling. Whole body wash has been compared with neck skin or a combination of tissue samples (Jorgensen *et al* 2002) and there were no significant differences. Frozen and chilled samples were also compared in this study, revealing a drop in prevalence from 91% to 81% when samples were frozen.

Both direct culture and enrichment methods, which enhance the growth of bacteria in a broth medium, have been used to isolate *Campylobacter* from tissue samples. Incubation in an enrichment broth before plating onto solid media, selective for *Campylobacter*, should result in enhanced recovery rates, but the opposite effect was documented (Musgrove *et al* 2001) in a survey of crop and cecal samples. Enrichment in Bolton broth followed by plating on a *Campylobacter* specific medium detected only 63.3% *Campylobacter* from cecal samples, where direct culture detected 100% of samples were positive. Enrichment enhanced isolation from crop content, detecting *Campylobacter* in 99.2% of 128 samples, however only 95.3% of these samples were positive by direct culture. The authors speculate that enrichment of cecal content may result in *Campylobacter* being overgrown by the large number of other enteric bacteria that would also be favored by enrichment, because it is not a selective process. Direct culture also allows for quantification of the bacterial levels in samples, which is not possible when enrichment is used.

Antibiotic MIC determination can be difficult with *Campylobacter* because of the specific growth requirements for this organism. Since 1992 the E-test has been regarded as the standard for the industry for determination of MIC values for *Campylobacter* (Tenover *et al* 1992) and is used by NARMS (National Antimicrobial Resistance Monitoring System) for surveillance of antimicrobial resistance (NARMS: Enteric Bacterial Working Group 2003). It is easily interpreted after 24 hours, which is much better than disc diffusion methods that require 48 hours. The MIC values that distinguish between sensitive and resistant are not well established for *Campylobacter* and must be inferred from those for use of the drugs against similar organisms.

Molecular methods, which detect specific genetic material, are increasingly used to permit investigators to compare isolates from various sources and trace outbreaks or infections. PFGE has been used to distinguish isolates with greater discrimination than traditional serotyping methods (Tenover *et al* 1995,Wu *et al* 2002). Other molecular methods, such as PCR and genomic probes are also useful to reliably identify *Campylobacter* to species level. *C. jejuni* and *C. coli* are distinguished only by their ability to hydrolyze hippurate, which is not always a consistent test, and separation of species based on recognition of specific DNA fragments is more accurate (Eyers *et al* 1993, Fermer and Engvall 1999, Gonzalez *et al* 1997, Korolik *et al* 2001, Linton *et al* 1997).

An Australian study in 1995 used restriction endonuclease DNA profiles to distinguish *C. jejuni* isolates originating from cloacal swabs of commercial poultry (n = 120) and human isolates (n = 49) (Korolik *et al* 1995). There were 89 unique strains and poultry and human isolates were indistinguishable by the restriction endonuclease test, although the *C. jejuni* isolates could be distinguished from *C. coli*. A Southern blot method with a DNA probe specific for *C. coli* and *C. jejuni* did distinguish between human and poultry isolates. This probe produced patterns with either a single 18.5 kb fragment or a split into two fragments at 14 kb and 4.5 kb related to their binding potential for *Cla*I.

The single site was found in 71% of the human isolates, but only 22% of the poultry isolates, indicating that there is a genetic distinction between *C. jejuni* strains commonly isolated from poultry and those that are pathogenic for humans.

The genotypic diversity of *C. jejuni* was studied by weekly fecal sampling for 3 weeks prior to slaughter (Thomas *et al* 1997) and revealed that commercial chickens carried multiple genotypes of *C. jejuni* in the flock and often in individual chickens. PCR methods were recommended as the most reliable to trace outbreaks because of the capacity to detect individual strains, but a virulence gene had not then been identified and if one was found it would make tracing outbreaks simpler.

Southern blot hybridization techniques and PCR have been useful to detect *C. jejuni* in fecal material. *C. jejuni* has been detected by Southern blot methods in chicks less than 3 weeks of age, as well as in 18-day-old embryos by both methods (Chuma *et al* 1997). The organism could not be grown by conventional culture in either instance, but this lends credibility to vertical transmission.

PFGE, fatty acid profiles and biotypes of *Campylobacter* isolates from meat plants in Ontario were compared (Steele *et al* 1998) using *C. jejuni* (n = 110) from environmental surfaces as well as isolates from employee fecal samples. PFGE with *Sma*1 revealed 86 distinct PFGE types but the criteria for relatedness were not specified. When PFGE groups from both *Sma*1 and *Sa*1 were used, 98 groups could be distinguished for 110 isolates. PFGE discriminated most accurately and gave an indication of the diversity of *C. jejuni* populations.

Human and poultry isolates of nalidixic acid resistant *Campylobacter* isolated between 1996 and 1998 were compared by PFGE with *Sma*1 and 105 different profiles were identified (Wu *et al* 2002). The criteria for distinguishing these profiles were not presented, but 11 were found in both poultry (23% of isolates) and human (40% of

isolates). The authors' conclusion that 40% of human cases can be attributed to poultry is not justified based on the information presented, but is indicative of the tendency to confuse association with causation.

Molecular methods have also been used to distinguish integron-like structures associated with sulfonamide resistance that are detectable by PFGE. One type was detected in both poultry and human isolates (38% of strains) of *C. jejuni* from Ireland (Lucey *et al* 2000) suggesting a common cause that the authors speculate was recombinant events between *Campylobacter* and other bacterial species in large commercial livestock farms.

PFGE has been used to explore genetic diversity of *Campylobacter* isolated from fresh retail chicken and isolates from human illness (Dickins *et al* 2002) confirming extensive variability in isolates from both sources. The prevalence of *Campylobacter* in the poultry carcasses was 82% and the same PFGE strain tended to be found on carcasses purchased at the same time from the same store. PFGE patterns remained consistent over many passages of these organisms in the laboratory indicating that PFGE is a reliable means of linking an isolate with the source.

4.6 Prevalence of Antimicrobial Resistance in *Campylobacter*

Campylobacter is known to rapidly develop resistance to various antibiotics and through a variety of mechanisms (Engberg *et al* 2001, Taylor and Courvalin 1988b). A marked increase in resistance, especially to the fluoroquinolone antibiotics has been noted since the late 1980s in several European countries and ascribed to approval and use of these drugs in livestock production (Endtz *et al* 1991, Rautelin *et al* 1991, Reina *et al* 1992). These drugs have been withdrawn from use for livestock in several countries. This includes enrofloxacin, the only fluoroquinolone licensed for use in livestock in Canada. It was initially approved as a turkey egg-dip in Canada in 1987,

but was withdrawn in 1997 (Engberg *et al* 2001), though it was still available by veterinary prescription through controlled drug release.

As early as 1981, comparisons of the sensitivity of *C. jejuni* isolates from human patients with enteritis (n = 72) to 18 antimicrobials were reported compared with sensitivities of 12 isolates obtained from chicken feces (Svedhem *et al* 1981). The time period over which samples were collected and the sampling method was not specified. They found no difference between human and poultry isolates for resistance to erythromycin, which was the drug of choice for human therapy, with 93% and 92% of the isolates, respectively, below an MIC value of 4, but only 17 and 16% sensitive below an MIC value of 0.5. Though not reported as significant, 89% of human isolates were sensitive to nalidixic acid, when only 61% of the poultry isolates were below the MIC of 16.

The emergence of resistance was also investigated in Spain in 1992 (Reina *et al* 1992) by a retrospective evaluation of *C. jejuni* isolates (n = 614) from 1987 to 1991. There were no resistant isolates in 104 strains from 1987, and 30% of 154 *Campylobacter* strains isolated in 1991 were resistant to ciprofloxacin (MIC >4), while 32% were resistant to nalidixic acid. Fluoroquinolone resistance could not be associated with prior treatment with or exposure to these drugs and the transfer of resistance from strains of livestock origin was considered most likely. The prevalence of erythromycin resistance was also examined and remained stable at about 3.5% in *C. jejuni*, and was approximately 19% in *C. coli* isolates.

In 1995 (Velazquez *et al* 1995b) it was reported that between 31 and 34% of human isolates of *C. jejuni* in Spain were resistant to fluoroquinolones (ciprofloxacin, norfloxacin and pefloxacin) and all were cross-resistant to nalidixic acid. There was no resistance to erythromycin, but 43% were resistant to tetracycline. The level of resistance of *C. jejuni* to fluoroquinolones had not risen appreciably from earlier

reports, but concern over the widespread use of these drugs in the poultry industry was still the source of speculation that this was the source of resistant human strains.

In the Netherlands, by 1991 increased resistance of *C. jejuni* clinical isolates in humans was being noted and Endtz and associates (Endtz *et al* 1991) examined 883 strains collected between 1982 and 1989 from both human stool and poultry products. Resistance to ciprofloxacin in poultry strains rose from 0% in 1982 to 14% of 189 isolates in 1989. The human isolates from 1982 were also all sensitive to ciprofloxacin, but by 1989 11% of 233 isolates were resistant. The increase in levels of resistance was seen as early as 1987, when 6 to 7% of all isolates were resistant. The isolates from poultry products were obtained from the public health laboratory system and it was not specified whether these were collected as part of routine monitoring or as follow-up to disease investigations. The latter might be suspected and would be a source of bias because strains associated with human disease might be more virulent. The authors ascribed the increase in ciprofloxacin resistance to availability of enrofloxacin for veterinary use, which was licensed in the Netherlands in 1987, although norfloxacin was approved for human use for urinary tract infections in 1985. They considered it unlikely that human to human spread of *Campylobacter* would occur, so resistance must have emerged first in poultry and been transmitted to humans through the food chain.

The trend to increased resistance to fluoroquinolone antibiotics was also noted in Finland by Rautelin *et al* (1991) who compared 102 isolates of *C. jejuni* from 1978 to 1980 with 100 isolates from 1990. The resistance to erythromycin remained constant at 3% (MIC break point 8) but resistance to norfloxacin rose to 11% from 4%, and resistance to ciprofloxacin rose from 0% to 9%. There was an association with travel to other countries for the patients with resistant forms of *Campylobacter*. The authors' interpretation was that exposure to novel strains in other countries was the reason for this resistance although ciprofloxacin had been available in Finland for human

treatment since 1987, patients with resistant isolates did not have a history of pre-culture treatment.

In 1992, the Centers for Disease Control and Prevention (CDC) surveyed 332 *C. jejuni* clinical isolates and only 1 was resistant to ciprofloxacin (Tenover *et al* 1992). There were 2% resistant to erythromycin. Most isolates were obtained prior to treatment, so resistance to drugs was not anticipated, but concern in Europe was growing and the authors predicted that resistance would develop quickly in response to therapy.

The level of fluoroquinolone resistance in the United Kingdom remained lower than the rest of Europe in the early 1990's. A small study (Piddock 1995) showed that 37/64 retail chickens had *Campylobacter* isolates and none were resistant to ciprofloxacin, but of the 26 of 50 retail chickens that were from a non-UK source, seven were resistant to ciprofloxacin. This was prior to the approval of enrofloxacin for use in poultry in the UK and rapid development of resistance was a concern.

As late as 1998, when the initial results from the Danish antimicrobial resistance-monitoring program were released (Aarestrup *et al* 1998), none of the 97 isolates of *C. jejuni* from broilers were resistant to enrofloxacin, unlike those found in most other European countries. Resistance was more prevalent (17%) in *C. coli* isolates from swine and attributed to the wide use of the drug in swine since it was introduced in 1993. Use statistics for poultry were not available, but the implication was that it was relatively infrequent.

Monitoring resistance in the USA by NARMS included *Campylobacter* after 1997 (NARMS: Enteric Bacterial Working Group 2003). As early as 1999 NARMS reports indicated that of 535 *C. jejuni* isolates from human cases from the seven state public health laboratories reporting between 1997 and 1998, 18% were resistant to nalidixic acid, 13% to ciprofloxacin and 5% to erythromycin (Marano *et al* 1999).

A summary of the *C. jejuni* human isolates monitored by NARMS between 1997 and 2000 revealed 1018 *C. jejuni* isolates from human illness, of which 15% were resistant to fluoroquinolones and 3% were resistant to erythromycin (Gupta *et al* 1999). There was a rise in fluoroquinolone resistance from 12.9% in 1997 to 17.4% in 2000. For comparison, 180 whole retail chickens were purchased (no selection criteria reported) and 44% were positive for *C. jejuni* with 19 of 79 (24%) resistant to fluoroquinolone and none resistant to erythromycin. The E-test was used, but the MIC values used to separate sensitive from resistant isolates were not reported.

An increase in prevalence of ciprofloxacin resistant strains of *C. jejuni* from 13% in 1997 to 20.5% in 1999 was also reported by NARMS (Rossiter *et al* 2001b). They compared this to an overall ciprofloxacin resistance of 24% in *Campylobacter* isolated from purchased chickens, but the method of culture and sampling for retail samples was not provided. The level of resistance did vary considerably from different areas of the USA, with highest prevalence of resistance in the northeastern USA and lowest levels in Georgia for poultry and for human isolates in Oregon. Unfortunately these levels of resistance were not compared with source of poultry or method of poultry production, because the highest poultry production is in southeastern states such as Georgia, and one would expect the most intensive agriculture might be associated with the highest level of prophylactic drug administration, which has been indicated to favor resistance development.

Rossiter *et al* (2001a) tested three retail chickens per month from three states between January and June 1999, from 25 stores representing 23 brands. They found 44% positive for *Campylobacter* and 19 of 80 were resistant to ciprofloxacin, 32% to nalidixic acid and 5% to erythromycin.

In addition to the higher prevalence of *Campylobacter* in broilers processed by immersion chilling relative to those chilled by air, antimicrobial resistance has been related to the chilling process used in slaughter facilities (Sanchez *et al* 2002). The *Campylobacter* strains isolated from immersion-chilled broilers were more resistant to fluoroquinolone antibiotics such as nalidixic acid than those isolated from air-chilled broilers. This may relate to different viability of resistant strains to drying, which is an influence with air chilling, or may reflect differences in management and drug treatment regimes used by suppliers to the two different types of slaughter facilities. There is a possibility that the processing methods may exert selective pressure and change antimicrobial resistance profiles of bacteria in the absence of drug use and this should be considered in trying to understand changes in resistance patterns over time.

4.7 Mechanisms of Antimicrobial Resistance in *Campylobacter*

The mechanisms by which *Campylobacter* develop resistance to various antibiotics have been reviewed by Taylor and Courvalin (1988b). Quinolone resistance has been strongly associated with inhibition of the DNA gyrase subunit A. This is substantiated by research of Segreti *et al* (1992) who used two clinical isolates of ciprofloxacin resistant *C. jejuni* and selected for this resistance over multiple generations. They were able to isolate the DNA gyrase holozyme and show it was 8 to 16 times less sensitive to inhibition by ciprofloxacin than the enzyme of the original isolate. This single step mutation was stable without any selective pressure, so it would remain in a population over time. The mutation in the subunit A of DNA gyrase has been confirmed to be at position 86 (Piddock 1995), which is the same site as fluoroquinolone resistance in other bacteria. The mutation was confirmed to be from ADA (threonine) to ATA (isoleucine) in resistant isolates studied as part of NARMS monitoring (Zirnstein *et al* 1999), but while this mutation is effective, it may not be the only mutation that can have this effect.

Quinolone resistance was induced during clinical treatment by Wretlind *et al* (1992b) in Sweden during a multi-centre, double-blind placebo controlled trial treating enteritis with norfloxacin. *C. jejuni* strains sensitive to norfloxacin were cultured from 15 of 60 patients at the start of the trial and eight of these patients received norfloxacin. Three of them developed resistant strains within 90 days post-treatment, and in one individual, the resistant strain was present at 4 days, during treatment. Seven patients positive for *C. jejuni* at the start of the trial received the placebo treatment and four of these were seen for follow-up. Three of them had norfloxacin sensitive strains of *C. jejuni* isolated. Resistant strains were not found in the placebo group. Though the numbers of patients are small, this documents the rapid appearance and persistence of fluoroquinolone resistant strains of *C. jejuni* after treatment.

Quinolone resistance has been experimentally induced in chickens (Jacobs-Reitsma *et al* 1994c) where six groups of 15 broiler chickens each were infected with quinolone sensitive *C. jejuni* and were subjected to treatment with enrofloxacin or flumequine. Enrofloxacin resistant *C. jejuni* could be cultured from all chickens treated with enrofloxacin regardless of dosage. These isolates were also resistant to flumequine (a second generation fluoroquinolone). The *C. jejuni* isolated from other treatment groups and control chickens remained sensitive. Treatment of chickens with enrofloxacin for the first 4 days of life, followed by *C. jejuni* inoculation did not induce resistance, indicating that early preventative therapy is unlikely to induce resistance in the gut microflora that can be transmitted to *C. jejuni* when it infects chicks. This is also a good indication that *C. jejuni* was not present subclinically in these chickens before they were intentionally infected for the trial.

4.8 *Campylobacter* Risk Assessments

Mathematical models have been used to predict the impact of *Campylobacter* from poultry sources and of fluoroquinolone resistant *Campylobacter* infections on human health. A comprehensive risk assessment that evaluated the prevalence of

fluoroquinolone resistant *Campylobacter* estimated that 50 to 70% of the 2 million cases of *Campylobacter* enteritis in 1998 could be attributed to chicken consumption (Hollinger *et al* 2001). Of those seeking medical care, 55% would likely have received a fluoroquinolone antibiotic. Based on the level of fluoroquinolone resistance in *Campylobacter* isolates from poultry, their report estimates that 5000 individuals a year might be ill with fluoroquinolone-resistant *Campylobacter* infections that would compromise their treatment. The degree of uncertainty around each step in the model is carefully considered.

Hartnett *et al* (2001) prepared a risk assessment to predict how likely a chicken selected at the time of slaughter from Great Britain's national flock would be positive for *Campylobacter*. The model considered exposure of the first chicken in a barn, the spread of the bacteria among chickens in a cluster and the spread between clusters to establish what the prevalence within a flock would be based on when the agent was introduced. The model predicted that delay in first exposure would have the most effect on reducing the prevalence in chickens going to slaughter. The model predicted a risk of 0.53 based on a flock starting with a single positive chicken. The model ignored vertical transmission. The probability of inter-chicken contact and the contact between chickens in adjacent clusters was based on estimates from experts that were quite divergent and there was nothing to account for spread within the barn by means other than direct chicken-to-chicken contact. Certainly the movement of bacteria within a flock, either through flowing water or by being tracked on manure-laden boots of farm workers would contribute to spread more rapidly and extensively.

These risk models are based on so much uncertainty that the conclusions lack credibility. The real potential of this tool is in the sensitivity analysis, through which the given variables can be examined through their range of possibilities. Those that have a major impact on the outcome can be identified. This could establish points that will be most responsive to intervention.

CHAPTER 5

Materials and Methods

5.1 Study Design and Sampling

Two cross-sectional studies were conducted during consecutive 8-week periods, sampling one production cycle of broiler chickens and a comparable number of whole retail chickens.

5.1.1 Study A: Isolation of *Campylobacter* spp. from Ceca of Chickens

The commercial broiler chicken population in Alberta is approximately 6 million chickens in every 8-week period. The estimate of population *C. jejuni* prevalence was made based on samples collected at four slaughter facilities rather than sampling during other phases of production, such as collecting fecal samples or chickens from the farms. This decision allowed for the efficient collection of the whole cecum of the intestine from freshly killed chickens, because the intestinal tract provides a high recovery of the bacteria (Berrang *et al* 2000, Jeffrey *et al* 2001) and the cecum is a descrete organ that is readily recognized for consistent collection. Sampling at the slaughter facility allowed representative sampling of a large population of chickens without extensive travel to multiple farms. Prevalence tends to rise with age during the life of a broiler chicken, so sampling at slaughter age would give the highest estimate of prevalence for the life of a broiler chicken. Chickens arriving at the slaughter facility are also stressed from transport, so they would be likely to have higher numbers of enteric bacteria and these may have been transmitted among chickens. The slaughter facility is also the last point for the sampling of live chickens whose gut content may be the source of bacterial contaminants on chicken meat.

The four federally inspected poultry slaughter facilities in Alberta process approximately 6 million broiler chickens every 8 weeks. One 8-week cycle was chosen

to represent the provincial broiler chicken population and sample size was calculated based on the provincial broiler chicken population during that time period. Based on weekly slaughter volumes in early 2001, 16% of these chickens were slaughtered at each of 2 small facilities and 34% at each of 2 larger facilities. The total sample size was allocated among the facilities in proportion to the slaughter volume.

A random sample of 370 chickens was required to establish the prevalence of *C. jejuni* with accuracy of 5% at 95% confidence (Appendix A). This was based on an estimated prevalence of 50% to use maximal variance. The sample size calculation was based on the same expected prevalence of 50% for *C. jejuni* as it would have been in sampling for combined *Campylobacter* species because *C. jejuni* is the species found almost exclusively in poultry. The range of reported prevalence is also extremely wide for both *Campylobacter* as a whole and *C. jejuni*, (from 30 to 99% for either) so the estimate for *C. jejuni* could not be more accurate than 50%. It was technologically feasible for the designated laboratory to process 400 cecal samples for *C. jejuni* culture over 8 weeks (50 samples per week). This estimate was based on the technical manpower to collect the samples from the slaughter facility, and allowed for transportation time from regional collection points to preserve samples for culture, as well as the technical manpower and facilities for culturing samples. Laboratory staff and time were available for samples to be collected in February and March 2001.

For the sample to be representative of the Alberta broiler chicken population, the samples for culture would need to be collected at random. Randomized designs based on units of slaughter time, slaughter days and producer identifier were not economically feasible and would jeopardize the validity of culture results because of the sample deterioration from excessive delays between tissue collection and bacterial culture if sampling were distributed randomly to regional slaughter facilities because this would require sample storage over weekends. It was not economically feasible to hire technical staff to cover all the hours of slaughter operation (early mornings and

weekends) for random sampling to be possible. It was also important to allocate samples according to slaughter volume, which took precedence over complete randomization.

The designated microbiology laboratory would not initiate culture on weekends, so sampling had to be coordinated between the slaughter facilities in Calgary and Lethbridge, with the two facilities in Edmonton, to ensure samples arrived at the laboratory within 24 hours of being collected on a weekday when culturing could be initiated. The slaughter facilities in Calgary and Lethbridge did not kill poultry every day, so they were sampled on days early in the week when poultry slaughters were scheduled. Samples from the two facilities in Edmonton were collected to equalize the number of specimens arriving in the laboratory on a daily basis.

A slaughter facility was visited once each sampling day. It would have been preferable to randomize sampling times throughout the selected day, but this was not practical. The samples for each day were collected with a minimum of 10 minutes between samples. This ensured that approximately 1200 carcasses would have passed between samples. The farm name was not collected with the samples, because of Freedom of Information Policy restrictions, but the technicians noted that approximately half the samples on any given day represented a single producer and the remaining samples were typically from two producers, and rarely from three or more.

At intervals of at least 10 minutes, a technician wearing disposable gloves selected a chicken arbitrarily from the processing line after evisceration, when Canadian Food Inspection Agency inspectors were evaluating the internal organs. As the intestinal tract was removed from the carcass, the paired ceca were identified and detached from the colon with sterile scissors. They were sealed in a sterile plastic bag (WhirlPac® Fisher Scientific) and placed in a cooler on ice packs. The technician changed gloves and instruments before selecting the next chicken. In one slaughter facility, the

company technicians were trained to perform the sample collections because of company policy that restricted admission of non-employees to the slaughter area.

5.1.2 Study B: Isolation of *Campylobacter* spp. from Chickens Purchased in Retail Stores

The population of chicken in the retail market was defined as whole, fresh, never-frozen broiler chickens offered for retail purchase by major, urban, grocery retailers during an 8-week interval in April and May 2001. There were between 50 and 60 major grocery outlets, identified through the telephone book, in each of Calgary and Edmonton, and eight grocery retailers in Lethbridge at the time of the study. A sample of 400 chickens could be processed at the designated laboratory over an 8-week period, and if random, this would provide a prevalence estimate that was accurate within 5% with 95% confidence. A random sample of 50 retailers each was chosen based on a random numbers table, from the telephone listings, for Calgary and Edmonton. When some stores did not have the required number of samples, four retailers were selected randomly for re-sampling to achieve the required 400 samples. All major grocery retailers in Lethbridge were sampled once each because staff and funds were available to increase the overall sample size and examine possible differences between major cities and a smaller centre.

Each Edmonton and Calgary retail store was visited twice and two chickens were selected at each sampling each time. Each store in Lethbridge was sampled once, selecting two chickens on each visit. Travel time, coordination and technical time made it impractical to sample a single chicken on each visit to a single store, so the clustering that resulted from sampling two chickens per visit was unavoidable. The stores were sampled by convenience , to ensure all chickens were purchased before noon and either cultured or shipped for culture on the same day. The chickens were selected from all whole broiler chickens on display and the fact that they were fresh, never frozen, was confirmed by asking the store staff. Individual chickens were selected randomly as the

first two chickens for which the last digit of the price matched the sequential numbers in a random number table. They were held on ice or chilled during transport to the laboratory for culture. Shipping took place overnight from Calgary and Lethbridge so samples were cultured within 24 hours of collection.

5.2 Laboratory Methods

5.2.1 Bacterial Isolation

All bacterial isolation, identification and confirmatory testing by PCR and PFGE was done by the staff of the Agri-Food Laboratories Branch, Alberta Agriculture, Food and Rural Development. A single laboratory was chosen for consistency in methods and availability as well as willingness to participate in the research. The laboratory is ISO17025 certified and the methods and media for isolation and identification of *C. jejuni* were consistent with those recommended by the Canadian Food Inspection Agency (CFIA) reference laboratory. Positive and negative control cultures were analyzed on an ongoing basis as part of routine quality control.

5.2.1.1 Study A: Isolation of *Campylobacter* spp. from Ceca of Chickens

The ceca of the chickens were transported on ice to the laboratory to be cultured within 24 hours of removal. Each cecum was incised with a scapel blade and the mucosal surface was swabbed and inoculated directly on selective Karmali agar culture plates (Oxoid, Nepean, ON) (Nadeau *et al* 2002) and into Bolton enrichment broth (Oxoid) as recommended by the CFIA reference laboratory. After 24 hours of incubation, the Bolton broth was streaked onto a Karmali agar plate. The first 100 cecal samples were subcultured after 48 hours incubation, but this was discontinued because all positive samples were detected at 24 hours. On occasion, samples that had been positive in Bolton broth at 24 hours were negative at 72 hours, so the subculture at 48 hours did not enhance isolation and this step was eliminated after the first 100 samples. The failure of the broth technique to maintain the bacterial growth from 24 to 48 hours may relate to

changes in the pH of the broth over time, due to the bacterial growth. Continuing the method without demonstrable value could not be economically justified.

All incubation of agar plates and broth media for the isolation of *C. jejuni* was done at 42°C under micro-aerophilic conditions of 5% O_2 and 10% CO_2 in anaerobic jars with *Campylobacter* Gas Generating Kits (Oxoid). Plates were enumerated after 72 hours incubation and suspect colonies were screened by Gram stain. All presumptive *Campylobacter* spp. were identified and confirmed with biochemical testing including oxidase, catalase and hippurate test reactions, because *C. jejuni* is the only *Campylobacter* species positive for hippuricase production (Koneman *et al* 1997, On *et al* 1996, Hald *et al* 2000). Preliminary species determination was based on Kirby Bauer sensitivity tests to nalidixic acid 30 and cephalothin 30 (Koneman *et al* 1997). Any presumptive positive cultures that could not be identified to species level with biochemical testing were confirmed by PCR testing for detection of hippuricase gene as well as specific genes for *C. jejuni*, *C. coli* and *C. lari*. Confirmed cultures were frozen in aliquots of whole sheep blood (Oxoid) and stored at -70°C for PFGE.

5.2.1.2 Antibiotic Sensitivity Testing

Antibiotic sensitivity testing to establish Minimum Inhibitory Concentrations (MIC) for nalidixic acid, ciprofloxacin, enrofloxacin and erythromycin was performed by an E-test (AB BIODISK®, Solna, Sweden). This is a manual, quantitative method of determining the level of antibiotic required to inhibit growth of the bacteria. Overnight growth of the organism on a blood agar plate was suspended in tryptic soy broth to achieve a turbidity of 0.5 McFarland. This suspension was swabbed onto a Mueller-Hinton agar plate (Oxoid) containing 5% sheep blood (Oxoid). Antibiotic impregnated strips were applied to the agar surface and the plates were incubated at 37°C in a micro-aerophilc atmosphere for 48 hours. The strips have the graded concentration of antibiotic printed along their length and the MIC was determined from the point on the strip where the zone of bacterial growth inhibition appeared.

5.2.1.3 Study B: Isolation of *Campylobacter* spp. from Chicken Purchased in Retail Stores

Purchased carcasses were rinsed with 100 ml sterile water and an aliquot of the wash was used for direct plating on Karmali agar as well as inoculated into Bolton enrichment broth. The broth was subcultured at both 24 and 48 hours because *C. jejuni* isolates were consistently recovered from the retail samples at both times. Culture and MIC procedures were identical to those used for the cecal samples.

5.2.2 Confirmation of *Campylobacter* spp. by Polymerase Chain Reaction (PCR)

The *Campylobacter* isolates requiring PCR confirmation were suspended in 12 mM Tris buffer (pH 8.0) and boiled for 10 minutes to break down the bacterial cells and release the bacterial DNA (template DNA). This was placed immediately on ice to prevent DNA degradation and PCR was performed that day. A 10 µL aliquot of this suspension was added to 40 µL of a PCR reagent mix (Invitrogen, ON) specific for *Campylobacter*. The final volume of 50 µL consisted of 1X PCR buffer to maintain pH 8.4, 2.5 to 3.5 mM magnesium chloride (concentration dependent on the primer pairs used), dNTPs (deoxynucleocide triphosphates dATP, dCTP, dGTP and dTTP), 0.02 U/µL Platinum® Taq polymerase and 0.2 to 1.0 µM primer pairs. All reagents were purchased from Invitrogen (Burlington, ON). The primers were specific for sequenced *Campylobacter* genes (Table 5.1), with one primer pair to confirm that a thermophilic *Campylobacter* was present, two different primer pairs specific for *C. jejuni* genes, one pair specific for *C. coli* and one pair specific for *C. lari*.

Table 5-1 The PCR primers used to identify and distinguish species of *Campylobacter*

Organism	Gene targeted	5' to 3' sequence	Product size *
Thermophilic *Campylobacter* spp. [a,b]	23S rRNA gene (Camp 1 & 2)	Forward-TATTCCAATACCAACATTAGT Reverse- CTTCGCTAATGCTAACCC	491 bp
C. jejuni [c]	*CeuE* (Camp 7& 8)	Forward- CCTGCTACGGTGAAAGTTTTGC Reverse- GATCTTTTTGTTTTGTGCTGC	987 bp
C. jejuni [d]	Hippuricase gene (Camp 3 & 4)	Forward- GAAGAGGGTTTGGGTGGTG Reverse- AGCTAGCTTCGCATAATAACTTG	735 bp
C. coli [c]	*ceuE* gene (Camp 9 & 10)	Forward- ATGAAAAAATATTTAGTTTTTGCA Reverse- ATTTTATTATTTGTAGCAGCG	894 bp
C. lari [a]	23S rRNA gene (Camp 1 & 6)	Forward- TATTCCAATACCAACATTAGT Reverse- ACGGCATCAGCAATTCTC	260 bp

[a] (Eyers *et al* 1993), [b] (Fermer and Engvall 1999), [c] (Gonzalez *et al* 1997), [d] (Linton *et al* 1997)
* in base pairs (bp)

The 50 µL aliquot of the template DNA and PCR reagents was inserted into the PCR thermocycler (PTC-200, Peltier Thermocycler, M.J. Research, Incline Village, Nevada) where it was heated to 94°C for 3 minutes to denature the double strands of DNA into single strands. The temperature was dropped to between 48 and 66°C (depending upon the primer pair) to allow the template DNA from the unknown bacteria to anneal with the DNA primers. The Taq polymerase bound to the short segment of primer/template double-stranded DNA. The temperature was raised to 72°C, the optimal temperature for Taq polymerase activity. During the polymerization, dNTPs were bound to the remainder of the template DNA strands, effectively duplicating the original template DNA present in the sample. The cycle of denaturation/annealing/elongation was repeated in the thermocycler resulting in amplification of specific recognized DNA, by doubling the amount of template DNA in each cycle. After 35 to 40 cycles, the original template DNA would be amplified to quantities that could be visually detected by electrophoresis of a 15 µL aliquot on a 1.2% agarose gel (Invitrogen) at 120 v for 30 minutes and staining with ethidium bromide (Invitrogen). Under ultraviolet illumination, the size of the PCR product was compared with a 100 bp ladder (Invitrogen) as a reference to confirm the identification of the isolate.

5.2.3 Pulsed-field Electrophoresis (PFGE)

All isolates of *Campylobacter* were fingerprinted by PFGE in a CHEF-DRIII® unit using the PulseNet protocol of the Center for Disease Control (Atlanta, GA). *Salmonella enterica* serovar Newport am01144 was used as the reference strain for profile analysis. Each *Campylobacter* isolate was embedded in agarose and lysed with N-lauryl sarcosine (Invitrogen) and proteinase K (Invitrogen) digestion. A thin slice of the embedded isolate was subjected to restriction endonuclease digestion with *Sma*1 (Invitrogen) to cleave the chromosomal DNA of each isolate into fragments. A thin slice of the agarose plug containing the isolate's chromosomal fragments was embedded in an agarose gel that was subjected to electrophoresis, with the current direction alternating through 120 degrees. The DNA restriction fragments were migrated through the agarose under the influence of the current and were resolved into a pattern of DNA in distinct bands relative to their molecular weight. This fragment pattern, specific for each isolate, was stained with ethidium bromide, viewed under ultraviolet light and then photographed for analysis of the band patterns.

Analysis and comparisons of the resulting fragment fingerprints were done with BioNumerics® (Applied Maths, S-Martens-Latem, Belgium) data analysis software. This program calculated the Dice coefficient, which is a similarity index that is an estimate of the proportion of the restriction fragments of the same size and position that were shared by two isolates (Dickens *et al* 2002, Nadeau *et al* 2002). This statistic is the result of a cluster analysis that reflects the band position and gives enhanced weight to matching bands over disparate bands. The tolerance, the amount of difference in band position that will be considered a match of the bands, was set to 1.5%. Bands more than 1.5% of the total pattern length apart were considered distinct.

5.3 Data and Statistical Analysis

Data were entered in and merged among Excel spreadsheets and analyzed with SPSS® statistical software. Descriptive statistics including the mean, range and interquartile range were carried out to summarize findings for individual continuous variables such as chicken weight and MIC for antibiotics. Odds ratios with 95% confidence intervals were calculated from two by two tables and stratified in three-way comparisons for covariates including slaughter facility, city, weekday of sampling, retail store, chicken brand and weight with isolation of *C. jejuni* from either cecal content or retail chickens and with the resistance of these isolates to various antibiotics. Fisher's Exact test was used when expected numbers were less than five. The variables with a significant relationship, as well as those that were predicted, based on biologically plausible association, to be potential confounders were incorporated into logistic regression models. The logistic regressions were performed for the slaughter and retail populations independently.

The relationship among the MIC for various antibiotics among isolates from cecal and retail sources were investigated by examining Spearman's correlation coefficients because the MIC variable was not normally distributed and had unequal variance, requiring the use of a non-parametric test for correlation. The associations were confirmed with Pearson's correlation coefficient when the MIC variables had been log transformed.

The comparison of the MIC concentration between groups of isolates required the use of the Mann-Whitney U-test when samples sizes and variance were unequal and the data was not normally distributed even when log transformed. This was often the case where comparisons between sub-groups resulted in relatively small sample sizes due to stratification of the sample, such as comparisons among brands, retail stores or slaughter facilities. The Mann-Whitney U-test provided a measure of whether the two groups were significantly different based on an analysis of rank, but does not allow for

an estimate of the difference between the means of the two groups nor a confidence interval.

A T-test was used for comparison of means when sample sizes were large and variances were equal, even if the distribution differed from normal, because the T-test is relatively robust under those circumstances. The T-test provided an estimate of the effect and a confidence interval and was used instead of the Mann-Whitney U-test to compare the log transformed MIC for the fluoroquinolone antibiotics.

The data on isolates of *C. jejuni* were analyzed in a discriminant analysis with logistic regression to investigate the relationship between antimicrobial resistance patterns and the PFGE types with slaughter and retail populations of *C. jejuni*.

5.3.1 Analysis of Antibiotic Resistance Data

The Minimum Inhibitory Concentration (MIC) values for antibiotic resistance were categorized into sensitive and resistant for enrofloxacin, ciprofloxacin and nalidixic acid based on category breakpoints as shown in Table 5-2. There were fewer than 5 isolates in the intermediate category for any fluoroquinolone antibiotic and these were grouped with resistant isolates for purposes of analysis. All three categories were used for erythromycin.

Table 5-2 MIC breakpoints for categorizing sensitivity and resistance of C. jejuni isolates to antibiotics

Antibiotic	Sensitive at MIC and below	Intermediate MIC range	Resistant at MIC and above
Ciprofloxacin	1	-	1.5
Enrofloxacin	0.25	-	0.38
Nalidixic acid	16	-	24
Erythromycin	0.5	0.75 – 4	6

The MIC concentrations reflect an exponential scale, because they are derived from serial dilutions of antibiotic concentration. This results in a much-extended scale for the MIC levels, with data results ranging from 0.047 to 256, with a frequency distribution centered on the values between 0.05 and 5. Results in this type of scale can be described and compared with median and interquartile range information. The original variables were used for comparison of medians between populations by Mann-Whitney U-test, which uses the ranks of the observations in the comparison to allow for the skewness in the data. The MIC variable was not treated as continuous for comparisons that are more complex because the few values at the extremes of the variable would have disproportionate impact on analyses. It is not appropriate to treat the variable as categorical in multivariate analyses because it is ordinal. To reflect the ordinal character and create a more appropriate scale, the MIC variables were log-transformed so that they could be more appropriately compared with the T-test and incorporated into a multivariate model.

5.3.2 Analysis of Pulsed-field Gel Electrophoresis Data

The BioNumerics® database analysis software generated a printout for each isolate of all *Campylobacter* species from both cecal and retail sampling that contained a visual representation of the individual isolate gel and a dendrogram that reflected the Dice coefficients (similarity coefficient). The Dice coefficient is a summary statistic based on a cluster analysis and illustrated by a dendrogram. The dendrogram is a branching diagram that positions the most similar isolates together and graphically indicates the percentage relationship among all isolates.

The comparison of PFGE patterns remains subjective, especially when an organism does not resolve into a large number of bands. Isolates are considered distinctly different when there is less than 50% similarity between the band patterns (Tenover *et al* 1995). Isolates are considered closely related if the genetic changes are consistent with a single genetic event that would result in fewer than three band differences. With

an organism like *Campylobacter* that has eight to ten bands, this would correspond to approximately 75% relatedness. The *C. jejuni* isolates were categorized accordingly. To investigate whether there were differences between the retail and cecal isolates with respect to membership in the more common PFGE categories, the infrequent categories, those with fewer than 10 isolates, were combined in one group, leaving the more common PFGE categories for analysis.

Campylobacter has only eight to ten PFGE bands compared with organisms like *Salmonella* that typically produce 40 to 50 bands in a gel. The lack of comparison points between gels means that a small difference between gels will have a proportionally greater impact on percentage relatedness than when many bands are present for comparison. Proportionally more *Campylobacter* isolates should be distinguished as unique than for an organism with many bands. In addition to evaluating membership in the major PFGE categories between cecal and retail isolates, it was important not to lose the information on the degree of relatedness among the more distinct isolates. This was captured through examining percentage relatedness of each individual isolate to selected reference strains.

Four strains of *C. coli*, two of *C. lari* and four of *C. jejuni* were selected to serve as reference strains to which all isolates could be compared. The strains were selected at random (case numbers matched to a random number table) from the most unique isolates (those with a single line on dendrogram and less than 60% related to any other isolates), such that there was at least one strain from each of the cecal and retail populations for each organism. The Bio Numerics® software calculated a relationship matrix for every *C. jejuni* isolate to the 10 reference strains. This was considered an interval variable for each isolate. These variables were evaluated graphically for co-linearity, by Spearman's rho for correlations and by a T-test for comparison of means between isolates from cecal and retail sources. These reference variables were then incorporated into the logistic model.

CHAPTER 6

Results

6.1 Study A: Isolation of *Campylobacter* spp. from Ceca of Chickens

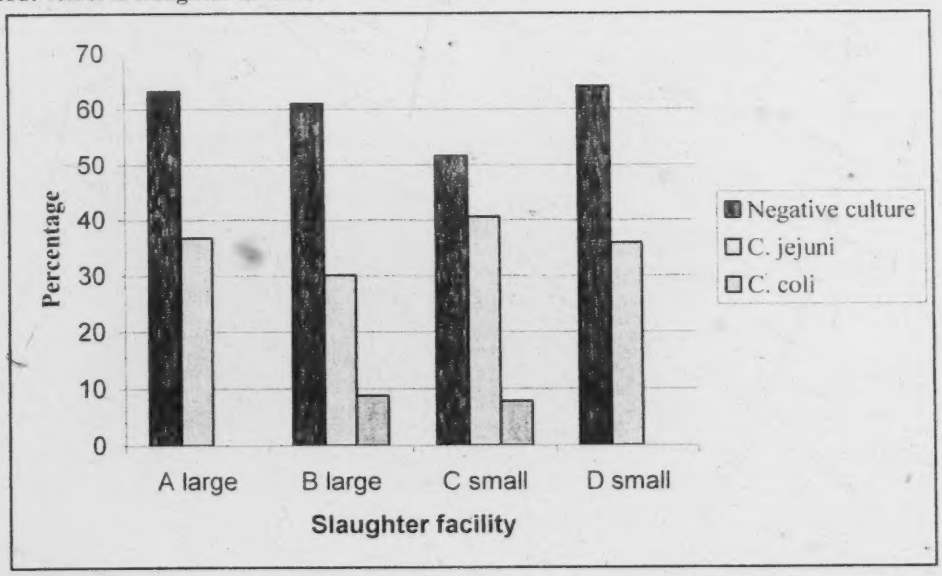
6.1.1 Results for Culturing *Campylobacter* spp. from Chicken Ceca

A total of 400 cecal samples were obtained from four slaughter facilities and by design these were proportional to slaughter volume, with 64 (16%) from each of two small facilities and 136 (34%) from each of two larger facilities. Of the 400 ceca, 243 were negative for *Campylobacter* species. *C. jejuni* was isolated from 140 ceca, *C. coli* from 17 ceca and *C. lari* was not isolated from any samples. The prevalence of combined *Campylobacter* in cecal samples from this sample of 6-week-old broilers at slaughter was 39.25%, the prevalence of *C. jejuni* was 35%, and the prevalence of *C. coli* was 4.25%. The distribution of *Campylobacter* culture results by slaughter facility is shown in Table 6-1. The percentage distribution of cecal culture results is illustrated in Figure 6-1.

Table 6-1 Distribution of *Campylobacter* culture results from broiler chicken ceca for the four Alberta slaughter facilities

Slaughter facility	Negative for *Campylobacter*		*C. jejuni*		*C. coli*		Total Samples
	#	%	#	%	#	%	#
A large	86	63.2	50	36.8	0	0	136
B large	83	61.0	41	30.1	12	8.8	136
C small	33	51.6	26	40.6	5	7.8	64
D small	41	64.1	23	35.9	0	0	64
Total	243	60.8	140	35.0	17	4.3	400

Figure 6-1 Percentage distribution of broiler chicken cecal *Campylobacter* culture results within the four Alberta slaughter facilities



The isolation of *C. jejuni* was the outcome of interest and was the variable considered in subsequent analyses. The odds of isolating *C. jejuni* were not significantly different among the slaughter facilities and the odds ratios (OR) and 95% confidence intervals (95% CI) for each slaughter facility relative to the first (A large) are shown in Table 6-2.

Table 6-2 Odds ratios (OR) and 95% confidence interval (95% CI) for isolation of *C. jejuni* from ceca of broiler chickens obtained from four Alberta slaughter facilities*

	OR (95% CI)	p-value
B large	0.85 (0.51, 1.42)	0.53
C small	1.36 (0.73, 2.52)	0.34
D small	0.97 (0.52, 1.79)	0.91

***reference category: A large**

The four Alberta slaughter facilities represented two companies and when grouped accordingly, the isolation frequency for *C. jejuni* were 36.4% and 36.8% for the

companies, respectively. The odds of *C. jejuni* culture did not differ significantly (OR: 1.01; 95% CI: –0.43, 0.44; p = 0.95) between the two companies.

Comparisons between the cities in which birds were slaughtered showed the odds of isolation of *C. jejuni* did not differ significantly among the three cities. While 44.1% (26/59) of birds slaughtered in Calgary were positive for *C. jejuni*, 35.0% (91/260) and 35.9% (23/64) of samples from Edmonton and Lethbridge, respectively, were positive for the presence of *C. jejuni*. Compared with Calgary, Edmonton had odds of *C. jejuni* isolation of 0.69 (95% CI: 0.39, 1.21) and Lethbridge had odds of 0.71 (95% CI: 0.34, 1.47). The comparison between Edmonton and Lethbridge gave OR: 1.04 (95% CI: 0.59, 1.84).

The day of the week on which the cecal samples were obtained had a significant impact on whether *C. jejuni* was isolated (Table 6-3). The odds of isolation of *C. jejuni* among the days of the week are summarized in Table 6-4.

Table 6-3 Distribution of *C. jejuni* isolation from ceca of chickens by weekday that the cecal sample was collected

Weekday of slaughter (cecal sample collected)	*Campylobacter* negative		*C. jejuni*		Total Samples
	#	%	#	%	#
Monday	49	67.1	24	32.9	73
Tuesday	47	51.6	44	48.4	91
Wednesday	45	60.0	30	40.0	75
Thursday	70	75.3	23	24.7	93
Friday	32	62.7	19	32.3	51

Samples collected on Tuesdays had marginally higher odds of being positive for *C. jejuni* than those collected on Monday (OR: 1.91; 95% CI: 1.01, 3.62; p = 0.05). They also had higher odds of being positive than samples collected on Thursdays (OR: 2.85; 95% CI: 1.53, 5.32; p = 0.0009). Samples collected on Wednesdays were also of higher

odds for positive culture than Thursday samples (OR: 2.02; 95% CI: 1.05, 3.93; p = 0.03).

Because the cecal samples collected from Calgary and Lethbridge required a day for transportation to the Edmonton laboratory for culture, the relationship between isolation of *C. jejuni* with city and weekday of sample collection was examined.

The initial examination of culture results relative to city showed the difference on Thursday was a result of Lethbridge submissions. The samples from Calgary relative to those from Edmonton have an OR: 1.11 (95% CI: 0.27, 4.71; p = 0.88) for positive culture. The comparison between Lethbridge and Edmonton on Thursday revealed an OR of 6.38, with Lethbridge more likely to produce a positive culture (95% CI: 2.05, 19.83; p = 0.0007).

Among samples collected on Tuesday, the OR for Calgary relative to Edmonton was 3.75 (95% CI: 1.08, 13.02; p = 0.03), while the OR for Lethbridge relative to Edmonton reverses this relationship, with OR: 0.3 (95% CI: 0.10, 0.88; p = 0.03). When day of week and city of origin were included in a logistic regression model, the effect of weekday when samples were collected remained significant (p = 0.025) when the city was controlled. The effect of the city was eliminated (Table 6-5), indicating that the day of week sampled was the only important predictor, regardless of transportation factors.

Table 6-4 OR and 95% CI for isolation of *C. jejuni* relative to weekday of sample collection from logistic regression model controlling for city and slaughter facility sampled*

	OR (95% CI)	p-value
Monday	1.57 (0.79, 3.12)	0.200
Tuesday	2.84 (1.52, 5.33)	0.001
Wednesday	1.98 (1.02, 3.86)	0.045
Friday	1.93 (0.89, 4.14)	0.090

***reference category Thursday**

6.2 Study B: Isolation of *Campylobacter* spp. from Chicken Purchased in Retail Stores

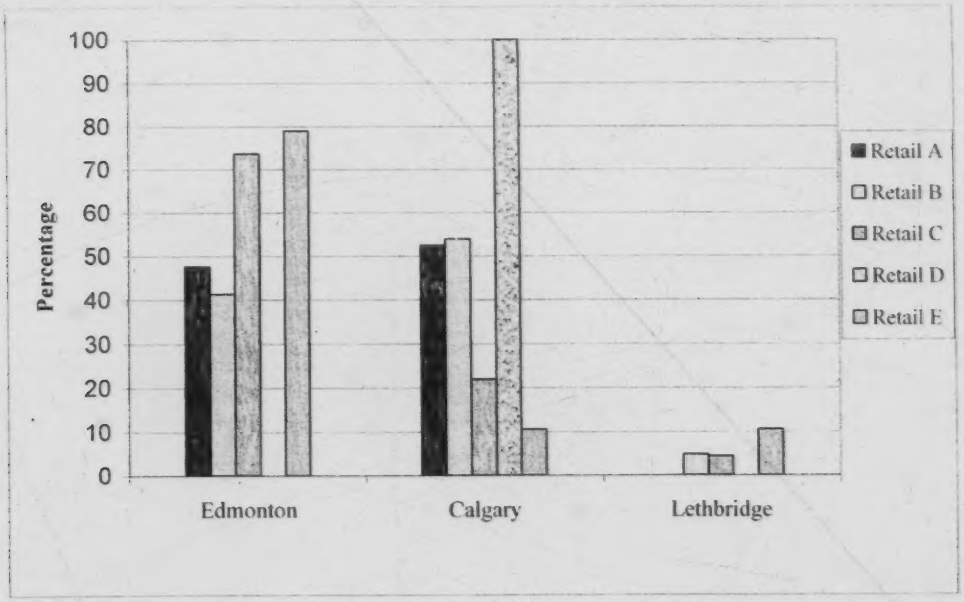
A total of 417 broiler chicken carcass samples were obtained from retail stores; 199 chickens from 50 Edmonton retailers, 202 chickens from 50 Calgary retailers and 16 chickens from 8 retailers in Lethbridge. The mean number of samples per retailer was 3.86 (range = 2 to 6, S.D. = 0.65). There was one store in each of Edmonton and Calgary that did not stock the whole broiler chickens after the first collection and one selected store in Calgary that closed prior to any samples being collected. On two occasions, in Calgary, five samples were taken from two stores and on three occasions, six samples were taken from one store to achieve the total number of samples required for culture. In Edmonton, one store provided five samples (in two sampling trips). Two samples were obtained from all eight retailers in Lethbridge.

The retail companies and chicken brands were not evenly distributed among cities but were a reflection of the retail stores and brands that were present in each city. There were five different retail companies in the three cities, distributed as shown in Table 6-5. It was worth noting that some retail stores were not present in some cities as illustrated by the graph of percentage distribution in Figure 6-2.

Table 6-5 Distribution of retail companies sampled for whole retail chickens by city (n = 417)

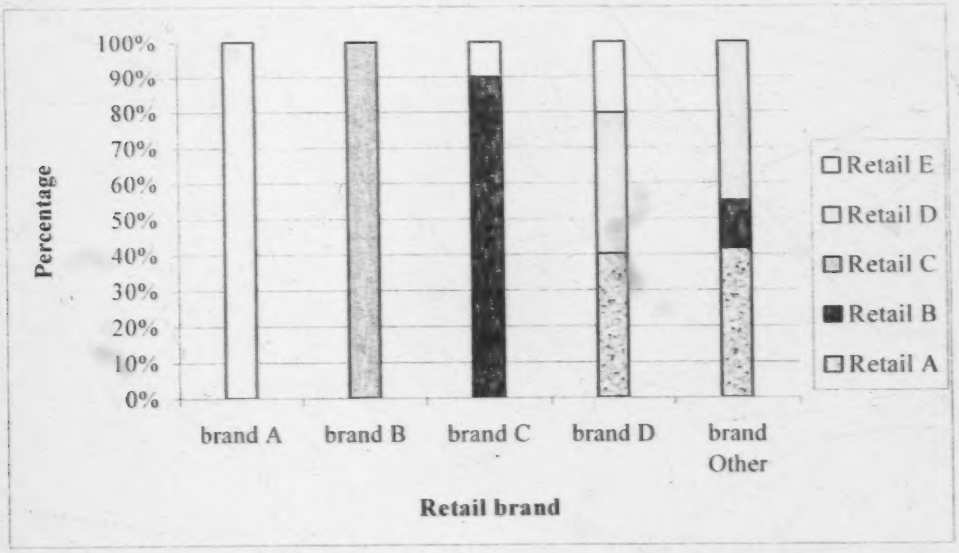
Retail store	Edmonton	Calgary	Lethbridge	Total
Retail A	20	22	0	42
Retail B	77	97	8	182
Retail C	70	27	4	101
Retail D	0	52	0	52
Retail E	32	4	4	40

Figure 6-2 Percentage distribution of retail companies sampled for whole retail chickens by city



There were seven brands of broiler chicken identified in the samples collected, four predominant brands with more than 15 samples each. Three chicken brands were infrequent, Brand E had 11 samples, Brand F had 9 samples and Brand G had 7 samples, so for analysis these were combined into a single category, representing 6.5% of all samples. Some brands were specific to a particular retail store and the brand distribution by retail store is shown in Figure 6-3. The relationship between brand and store was an indication that retail store and brand effects required a multivariate model to control for confounding.

Figure 6-3 Percentage brand distribution of whole retail chickens sampled by retail store

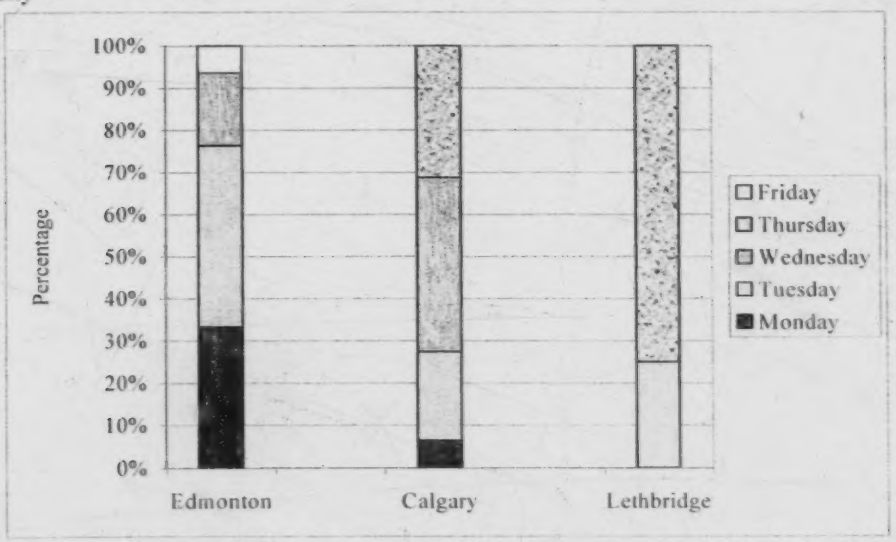


The weight distribution of retail chicken carcasses showed that 19 of 417 chickens were over 2 kg, putting them in the roaster category (chickens slaughtered at approximately 9 weeks of age) rather than broilers. While these samples might have been discarded, they do represent a portion of whole chickens available for consumer purchase, so they were included in the analyses relative to *C. jejuni* isolation findings. The roaster chickens over 2 kg were evenly distributed between samples from Calgary and Edmonton, but none was obtained from stores in Lethbridge.

When the weight categories were compared, the larger birds were associated with some stores and brands. No birds over 2 kg were obtained from Retailers C or E. There were 52.6% (10/19) roasters from Retailer A, 36.8% (7/19) from retailer B and 10.5% (2/19) from retailer E. None of the roasters was of brands A or B, two (10.5%) were Brand C, four (21.1%) were Brand D and 13 (68.4%) were from the group of infrequent brands designated Other.

The retail sample collection was distributed unevenly by day of the week relative to city of purchase, and the percentage distribution is shown in Figure 6-4. This was a function of study design to facilitate sample transport to the Edmonton laboratory so that culture was initiated during the week and to regulate the number of samples arriving at that laboratory on a daily basis, as well as to coordinate technical time between this project and others. The day of week in which samples were collected might reasonably be predicted to influence results of bacterial culturing because of how long chickens had been held in the retail display under variable temperature conditions since the time of slaughter. The potential influence of transportation time was also considered and the day of week that samples were collected had to be controlled for in the final model.

Figure 6-4 Percentage distribution of weekday of sampling for whole retail chicken samples in each city



6.2.1 Results for Culturing *Campylobacter* spp. from Chicken Purchased in Retail Stores

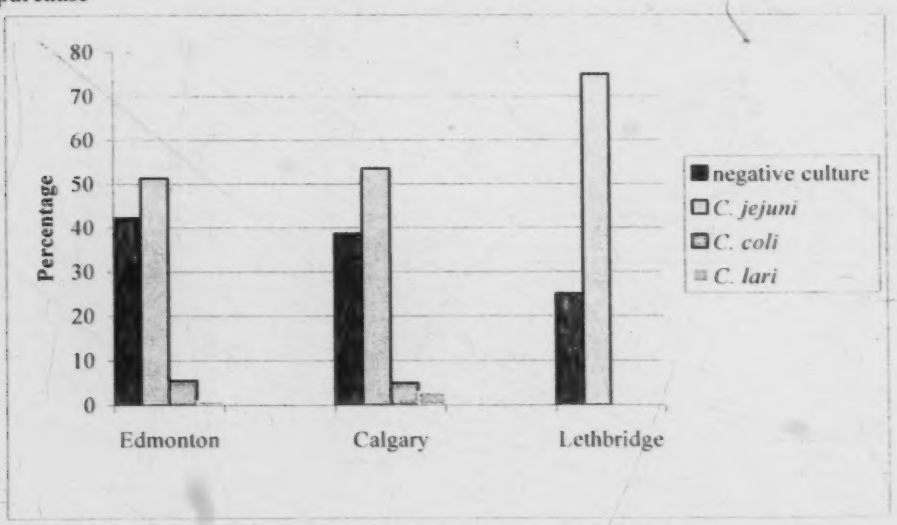
The culture of 417 whole body washes of retail chickens produced 222 isolates of *C. jejuni* giving a sample prevalence of 53.2%. There were 166 samples (39.8%) that were

negative for *Campylobacter* spp. *C. coli* and *C. lari* were isolated from 5% and 1.9% of retail samples, respectively. Complete culture results are presented in Table 6-6 and the percentage distribution of culture results by city is shown in Figure 6-5.

Table 6-6 Distribution of *Campylobacter* culture results from whole retail chickens by city where samples were purchased (n = 417)

Store City	*Campylobacter* negative		*C. jejuni*		*C. coli*		*C. lari*	
	#	%	#	%	#	%	#	%
Edmonton	84	42.2	102	51.3	11	5.5	2	1.0
Calgary	78	38.6	108	53.5	10	5.0	6	3.0
Lethbridge	4	25.0	12	75.0	0	0	0	0
Total	166	39.8	222	53.2	21	5.0	8	1.9

Figure 6-5 Percentage distribution of *Campylobacter* results for whole retail chickens by city of purchase



C. jejuni isolation was the outcome of interest. When Edmonton was considered as the baseline, the odds of *C. jejuni* isolation did not differ from Calgary (OR: 1.14; 95% CI: 0.76, 1.72; p = 0.53) but the OR for Lethbridge relative to Edmonton was 2.47. This was not significant and reflected the small sample size from Lethbridge (95% CI: 0.77, 7.94; p = 0.12).

In considering the retail company, compared with Retail A, there were no significant differences in the odds of isolating *C. jejuni*, except with Retail company E, which had OR: 0.35 (95% CI: 0.14, 0.87; p = 0.02). This indicated that this retailer had significantly lower odds of chicken testing positive for *C. jejuni* than chickens obtained Retail A, and all of the other retailers.

The retail brand of chicken, in comparison to the combined group of infrequent brands, showed significant differences among the brands as shown by the odds ratios in Table 6-8. The most common brands (B, C and D) do not differ from each other for the odds of isolation of *C. jejuni*. There were significant differences between each of the common brands and the less frequent Brand A as well as between each of them and the group of infrequent brands E, F and G (Other). Brand A had significantly lower odds of being positive for *C. jejuni* than any of the other brands had or the group of brands had. The group of infrequent brands had lower odds of a positive *C. jejuni* culture than any of the three frequent brands had. Because of the potential confounding effect among city, retail store, retail brand and day of week sampled, all these variables were considered in a logistic model.

Table 6-7 Comparison of retail brands for the odds for isolating *C. jejuni* from whole retail chickens

	OR (95% CI)	p-value
Brand A to other	0.19 (0.03, 0.99)	0.04
Brand B to other	2.40 (0.97, 5.93)	0.05
Brand C to other	2.07 (0.88, 4.89)	0.09
Brand D to other	2.81 (1.13, 6.97)	0.02
Brand A to Brand D	0.07 (0.01, 0.31)	<0.0001
Brand B to Brand D	0.85 (0.47, 1.56)	0.61
Brand C to Brand D	0.74 (0.43, 1.25)	0.26
Brand A to Brand C	0.09 (0.02, 0.41)	0.00015
Brand B to Brand C	1.16 (0.68, 1.96)	0.59
Brand A to Brand B	0.08 (0.02, 0.36)	<0.0001

In examining the relationship between the *Campylobacter* isolates and the chicken weight category (whether over 2 kg or below), only *C. jejuni* was isolated from the carcasses that weighed more than 2 kg. The odds of isolating *C. jejuni* from the roaster chickens did not differ from broiler chickens (OR: 1.03; 95% CI: 0.40, 2.62; p = 0.95). *C. coli* and *C. lari* were found only in the broiler chickens, but were such infrequent isolates that they would not have been expected from the low numbers of roasters sampled. The fact that *C. jejuni* was the outcome of interest and that the results for roaster chickens did not differ from those for the target broiler population, supports the decision to include the few roasters in the overall analysis.

The relationships among variables affecting the isolation of *C. jejuni* for the chicken samples obtained from retailers required three-way comparisons prior to establishing a multivariate model. These included examining the relationship between city, retail store and retail brand as well as examining the relationship between city and weekday of sampling. There was only one marginally significant relationship, between retailers relative to city sampled (and only the comparisons for Retail A and B between Edmonton and Calgary had sufficient numbers to produce expected values over 5) (Table 6-8).

Table 6-8 Comparisons of retailers by city for odds of isolation of *C. jejuni* from whole retail chickens

	OR (95% CI)	p-value
Retail A Edmonton to Calgary	0.278 (0.07, 1.03)	0.05
Retail B Edmonton to Calgary	0.91 (0.48, 1.71)	0.76
Retail B Edmonton to Lethbridge	2.18 (0.41, 11.56)	0.35
Retail B Calgary to Lethbridge	2.4 (0.46, 12.54)	0.29
Retail C Edmonton to Calgary	1.78 (0.61, 5.12)	0.29
Retail C Edmonton to Lethbridge	61.84 (0.01, ∞)*	0.09
Retail C Calgary to Lethbridge	34.88 (0.01, ∞)*	0.22
Retail E Edmonton to Calgary	2.33 (0.29, 19.24)	0.42
Retail E Edmonton to Lethbridge	2.33 (0.29, 19.24)	0.42

* the model was unable to fit the data because of the small sample size when stratified

The results of investigating retail brand relative to city for isolation of *C. jejuni* were only meaningful for common brands B, C, D between Edmonton and Calgary, and in none of these comparisons was the OR significantly different from 1. In other cases, the overall number of isolates and samples were too low for meaningful analysis. When city and day of week were examined together, there were no differences in the odds of *C. jejuni* isolation among cities, dependent on the day of week the samples were taken, but sample numbers were low. It was still important to retain this variable in a multivariate model because it may have a confounding or effect modifying impact.

A logistic regression model was created to establish the relationship between the isolation of *C. jejuni* and multiple possible predictors including weekday of sampling, retail brand, retail store and city. A component of each of these variables had been established as associated with the outcome of *C. jejuni* isolation through bivariate comparisons and there were relationships among the variables as well suggesting that those that did not have a significant impact on the outcome might still be confounding variables. A logistic regression model would illustrate the effect of each individual component of these variables, controlled for each of the other variables. The logistic regression with these categorical variables forced into the model showed that the weekday of sampling and the city did not impact the odds of *C. jejuni* isolation. Though weekday of sample collection was not an overall significant predictor of *C. jejuni* isolation, samples collected on Thursday, relative to Monday samplings, had significantly higher odds of being positive for *C. jejuni*, by a ratio of 2.77 (95% CI: 1.18, 6.51; $p = 0.02$). When controlled for weekday of sample collection, retail store and city, the retail brand was a significant predictor ($p = 0.035$) of *C. jejuni* isolation. The pool of brands (Other) did not differ from Brand A in the odds of *C. jejuni* isolation, but all other brands had substantially higher odds of being positive (Table 6-9). There was no difference among the retail stores that was not explained by the brand effect.

Table 6-9 Logistic regression model results of a model examining chicken brand, retail store, weekday and city of sampling in relationship to the isolation of *C. jejuni* from whole retail chicken in Alberta

Variable	OR	95% C.I. for OR		p-value
		Lower	Upper	
Monday *	1.00			
Tuesday	1.34	0.72	2.49	0.357
Wednesday	1.57	0.78	3.14	0.207
Thursday	2.77	1.18	6.51	0.02
Friday	1.25	0.35	4.52	0.735
Brand A *	1.00			0.035
Brand B	9.22	1.34	63.23	0.024
Brand C	7.45	0.89	62.03	0.063
Brand D	11.45	1.96	67.07	0.007
Brand Other	4.4	0.61	32.06	0.143
Retail A *	1.00			0.692
Retail B	1.1	0.25	4.84	0.90
Retail C **				
Retail D	1.31	0.53	3.22	0.56
Retail E	0.64	0.20	2.03	0.45
Edmonton *	1.00			0.12
Calgary	0.62	0.34	1.10	0.10
Lethbridge	1.77	0.42	7.53	0.441

* Reference category

** Redundant variable. Brand B was sold only at Retail C; therefore Retail C was eliminated from the regression because the effect was fully accounted for by Brand B.

6.3 Antimicrobial Resistance of *Campylobacter* spp. Isolated from Cecal Samples and from Chickens Purchased from Retail Stores

A total of 364 strains of *Campylobacter* spp. were tested for antibiotic resistance. All three fluoroquinolone antibiotics gave similar results among the *Campylobacter* species. All eight *C. lari* isolates were resistant to all three fluoroquinolone antibiotics. Of the 17 *C. coli* isolated from cecal samples, 11 (64.7%) were sensitive to all fluoroquinolones and six (35.3%) were resistant to all fluoroquinolones. Of the 21 isolates of *C. coli* from samples obtained from retail stores, all were sensitive to ciprofloxacin and nalidixic acid and one was resistant to enrofloxacin. The sensitivity results for *C. jejuni* isolates are presented in Table 6-10.

Table 6-10 Results of fluoroquinolone sensitivity/resistance categorization of cecal and retail source *C. jejuni* isolates (n = 362)

		Cecal isolates		Retail isolates	
		#	%	#	%
Enrofloxacin	Sensitive (MIC under 0.25)	122	87.14	195	87.83
	Resistant (MIC over 0.38)	18	12.86	27	12.16
Ciprofloxacin	Sensitive (MIC under 1)	125	89.29	204	91.89
	Resistant (MIC over 1.5)	15	10.71	18	8.11
Nalidixic acid	Sensitive (MIC under 16)	125	89.29	205	92.34
	Resistant (MIC over 24)	15	10.71	17	7.66

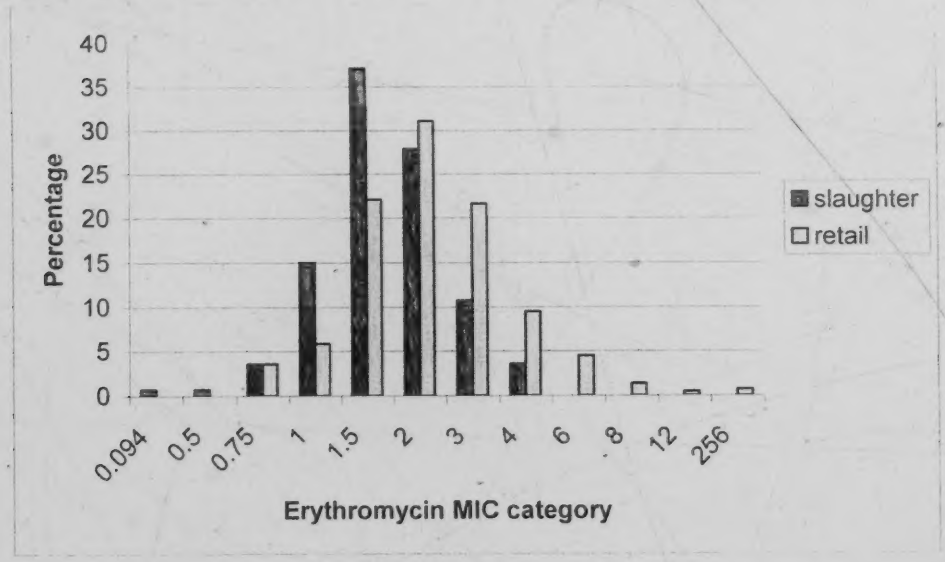
The medians, range and interquartile ranges for MIC to ciprofloxacin, enrofloxacin and nalidixic acid for C.*jejuni* isolates are shown in Table 6-11.

Table 6-11 Descriptive statistics of fluoroquinolone antibiotic MIC data for cecal and retail source isolates of *C. jejuni*

		Cecal	Retail	All isolates
Enrofloxacin	Median	0.125	0.190	0.125
	Range	31.95	31.93	31.95
	Interquartile range	0.031	0.133	0.096
	Minimum	0.047	0.064	0.047
	Maximum	32.00	32.00	32.00
Ciprofloxacin	Median	0.125	0.19	0.19
	Range	31.95	31.95	31.95
	Interquartile range	0.11	0.255	0.125
	Minimum	0.047	0.047	0.047
	Maximum	32.00	32.00	32.00
Nalidixic acid	Median	1.50	2.00	2.00
	Range	255.25	255.25	255.25
	Interquartile range	0.50	1.50	0.75
	Minimum	0.75	0.75	0.75
	Maximum	256.00	256	256.00

Evaluation of erythromycin sensitivity patterns for *C. jejuni* from cecal specimens showed that 97.9% (137 of 140) of the isolates were in the intermediate category, while 93.7% (208 of 222) of the *C. jejuni* from the retail specimens were intermediate. As a categorical variable, erythromycin MIC had insufficient variance to be a useful indicator, so the actual MIC was used in evaluating erythromycin resistance patterns. The percentage distribution for *C. jejuni* isolated from cecal and retail samples relative to the MIC for erythromyin is shown in Figure 6-6. This illustrates the difference between these two populations, with isolates obtained from retail samples tending toward a higher level of resistance than cecal isolates. These population differences were explored through comparison of means and medians for MIC to erythromycin and the fluoroquinolone antibiotics.

Figure 6-6 The percentage distribution of cecal and retail source isolates of *C. jejuni* by erythromycin MIC categories



The degree of correlation between the MIC for the fluoroquinolone antibiotics for all *C. jejuni* isolates by Spearman's correlation (nonparametric) is shown in Table 6-12.

Table 6-12 Spearman's correlation coefficient between the MICs for three fluoroquinolone antibiotics and erythromycin for isolates of *C. jejuni* from both cecal and retail sources (n = 362)

	Ciprofloxacin MIC	Enrofloxacin MIC	Nalidixic acid MIC	Erythromycin MIC
Ciprofloxacin MIC	1.000			
Enrofloxacin MIC	0.817*	1.000		
Nalidixic acid MIC	0.629*	0.684*	1.000	
Erythromycin MIC	0.493*	0.552*	0.478*	1.000

*** correlation is significant at 0.01 level**

The associations are even more strongly reflected by Pearson's correlation coefficient that was determined for the log MIC variable (Table 6-13). This indicates that one of the fluoroquinolone antibiotics could be chosen for multivariate models. While

resistance to erythromycin was significantly associated with resistance to fluoroquinolones, it is only a weak linear relationship with ciprofloxacin and not significantly associated with the other two fluoroquinolones.

Table 6-13 Pearson's correlation coefficient between the log transformed MICs of the three fluoroquinolone antibiotics and erythromycin for isolates of *C. jejuni* from both cecal and retail sources (n = 362)

	Log Ciprofloxacin MIC	Log Enrofloxacin MIC	Log Nalidixic acid MIC	Log Erythromycin MIC
Log Ciprofloxacin MIC	1.000			
Log Enrofloxacin MIC	0.965*	1.000		
Log Nalidixic acid MIC	0.957*	0.962*	1.000	
Log Erythromycin MIC	0.139*	0.099	0.080	1.000

*** correlation is significant at 0.01 level**

6.3.1 Antimicrobial Resistance Relative to Slaughter Variables

The antibiotic resistance information on *C. jejuni* isolates was of interest for comparison between slaughter facilities and companies as the source of *C. jejuni* isolates. The comparison of resistance to the fluoroquinolone antibiotics might be expected to differ because they are used clinically in poultry production and the poultry processing companies are often the source of treatment advice to poultry producers. The isolates from the samples obtained from the two companies were compared for resistance to each of the antibiotics, keeping in mind that enrofloxacin is the drug that has been used in poultry production.

The initial comparison was between the means of the MIC concentrations for the isolates obtained from chickens originating from the two companies. A Mann-Whitney U-test for comparing means was used because the sample sizes were unequal, MIC data were not normally distributed and variance was not homogeneous even when transformed with a log function. The results of the analysis are summarized in Table 6-

14. This test indicated that there was a significant difference between the means of the MIC for ciprofloxacin and was the difference was borderline for enrofloxacin and erythromycin, but there was no detectable difference for nalidixic acid. In all cases, isolates originating from company X were more resistant than those originating from company Y were.

Table 6-14 Results for the Mann-Whitney U-test analysis of the mean ranks of the MICs of four antibiotics for isolates of *C. jejuni* obtained from the two companies

Antibiotic	Company	N	Mean Rank	p-value
Ciprofloxacin	X	90	77.19	0.007
	Y	50	58.46	
Enrofloxacin	X	90	75.09	0.060
	Y	50	62.24	
Nalidixic acid	X	90	73.30	0.248
	Y	50	65.46	
Erythromycin	X	90	75.19	0.056
	Y	50	62.06	

The Mann-Whitney U-test was also used to examine differences among the four slaughter facilities, because the reduced sample size with each slaughter facility would affect distributions such that a T-test would not be robust. There were no differences between the two small slaughter facilities for any of the antibiotics. The facilities that differed are summarized in Table 6-15. Overall, isolates from slaughter facility A large were more likely to have a lower MIC for antibiotics than any of the other facilities.

Table 6-15 Results for the Mann-Whitney U-test analysis of the mean ranks of the MICs of four antibiotics for isolates of *C. jejuni* obtained from the four slaughter facilities

Antibiotic	Slaughter facility (n)		Mean Rank		p-value
			First facility	Second facility	
Ciprofloxacin	A large (50)	B large (41)	40.25	53.01	0.018
	A large (50)	C small (26)	34.28	46.62	0.017
Enrofloxacin	A large (50)	B large (41)	41.58	51.39	0.060
	A large (50)	C small (26)	35.36	44.54	0.071
Nalidixic acid	A large (50)	C small (26)	34.32	46.54	0.015
	B large (41)	C small (26)	30.15	40.08	0.034
Erythromycin	A large (50)	D small (23)	33.44	44.74	0.026

The relationship between resistance to enrofloxacin and the company was also examined considering enrofloxacin resistance as a categorical variable to establish the odds ratios, but the number of resistant isolates was very small and Fisher's exact tests revealed no significant differences.

6.3.2 Antimicrobial Resistance Relative to Retail Variables

The Mann-Whitney U-test was used to determine if there were differences among brands, stores, and cities for samples obtained from retail stores. There was little biological support for expecting a difference, or being able to explain one if it was found. *C. jejuni* isolates from retailers in Edmonton showed a significantly lower ($p = 0.015$) mean rank of MIC to nalidixic acid than those from Calgary retailers, but there were no differences among the MIC of the other antibiotics or with isolates obtained from samples collected from Lethbridge retailers.

The MIC for antibiotic resistance of *C. jejuni* isolates from the three most frequent retail brands, B ($n = 56$), C ($n = 94$) and D ($n = 60$) were compared and those of Brand B did not differ from those of Brand C for any MIC. Isolates from both Brand B and C had a significantly higher MIC to ciprofloxacin ($p = 0.001$) and erythromycin ($p = 0.02$) than those from Brand D. Isolates from Brand C also had significantly higher MIC to

enrofloxacin ($p = 0.019$) than Brand D. There were no significant differences between brands for *C. jejuni* resistance to nalidixic acid.

This was further investigated by examining the categorical resistance for the fluoroquinolone antibiotics by comparing these brands. The differences between isolates from Brand C and Brand D were eliminated for all antibiotic resistances when MIC categories of sensitive and resistant were evaluated with Fisher's Exact test of differences in proportions. However, Brand D was significantly less frequently resistant to ciprofloxacin ($p = 0.02$) and enrofloxacin ($p = 0.02$) than Brand B, and there was a detectable difference for the MIC of nalidixic acid ($p = 0.037$).

Comparison of MIC values by retail stores with the Mann-Whitney U-test showed that there were no differences among MIC values for *C. jejuni* isolates from retailers B, C and D, but there were some significant differences detected as shown in Table 6-16. *C. jejuni* isolates from retail store A had lower resistance to several antibiotics than those isolates obtained from retail stores B, C or D. Retail store E tended to have *C. jejuni* isolates that were less resistant to antibiotics. It is likely that these differences were a reflection of the retail brands that were carried in these stores and this testing confirms that the retail store and retail brand variables should be included in a multivariate model to distinguish between various *C. jejuni* isolates.

Table 6-16 Significant Results of Mann-Whitney U-test analysis to compare mean ranks of the MICs of four antibiotics for isolates of *C. jejuni* obtained from different retail stores

Antibiotic	Retail store (n)		Mean Rank		p-value
			First Store	Second store	
Ciprofloxacin	A (25)	B (96)	41.34	66.12	0.001
	A (25)	C (56)	29.68	46.05	0.003
	C (56)	D (32)	48.94	36.73	0.027
Enrofloxacin	A (25)	B (96)	43.74	65.49	0.005
	A (25)	C (56)	29.68	46.05	0.018
Nalidixic acid	A (25)	D (32)	22.84	33.81	0.010
	D (32)	E (13)	25.84	16.00	0.019
Erythromycin	A (25)	B (96)	42.24	65.89	0.002
	A (25)	C (56)	30.06	45.88	0.004
	A (25)	D (32)	23.00	33.81	0.011

6.3.3 Comparison of the Antimicrobial Resistance of *C. jejuni* from Cecal and Retail Samples

The comparison of MIC data between *C. jejuni* isolated from cecal and retail samples would help establish whether these organisms are from the same or different populations. The initial comparison was between the means of the MIC concentrations between the cecal and retail groups of *C. jejuni*. The log transformed MIC variable was used for a T-test because log transformation resulted in equal variances (by Laverne's test) and the T-test is relatively robust to the deviation from normal distribution when sample sizes are large. The T-test identified a significant relationship between erythromycin resistance and cecal or retail source of the *C. jejuni* isolate, giving a mean difference in log MIC of -0.115 (95% CI: -0.166, -0.064) which corresponds to cecal samples having an average MIC 1.12 units below retail isolates, with the 95% CI of that difference ranging from 1.06 to 1.18. The mean of the resistance to fluoroquinolone antibiotics was not significant different between the cecal and retail samples. While it may be useful to include the log MIC of all antibiotics in the multivariate analysis, this evaluation indicates that the degree of resistance to erythromycin was more likely to be

able to discriminate between isolates from cecal and retail samples than the resistance to fluoroquinolone antibiotics.

6.4 Results of Pulsed-field Gel Electrophoresis Analysis

6.4.1 Results of Categorical Relatedness by PFGE

Only three of the 361 *C. jejuni* isolates would not produce a PFGE gel pattern. The isolates produced between six and ten bands in the final gel. The 358 isolates that had PFGE patterns ranged from indistinguishable (100% similar) to unique (less than 40% related). Examples of the PFGE patterns are shown in Figure 6-7 and an example of the dendrogram generated by the computer analysis is shown in Figure 6-8.

Figure 6-7 Pulsed-field gel electrophoresis patterns of reference strains (wells 1, 8 and 15) and multiple *C. jejuni* isolates (remaining wells).

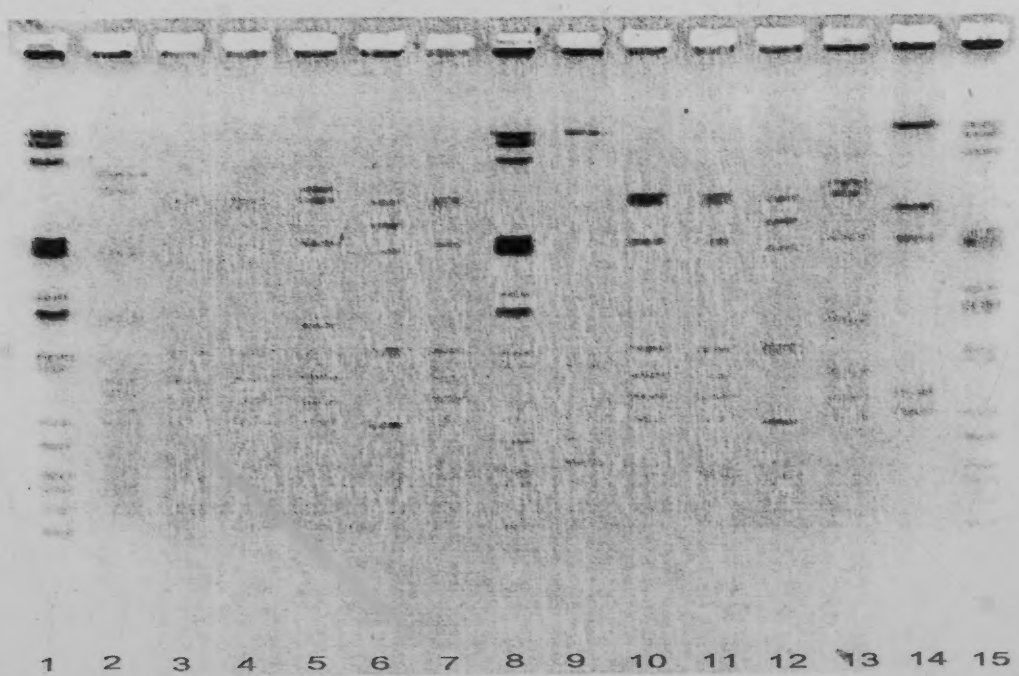
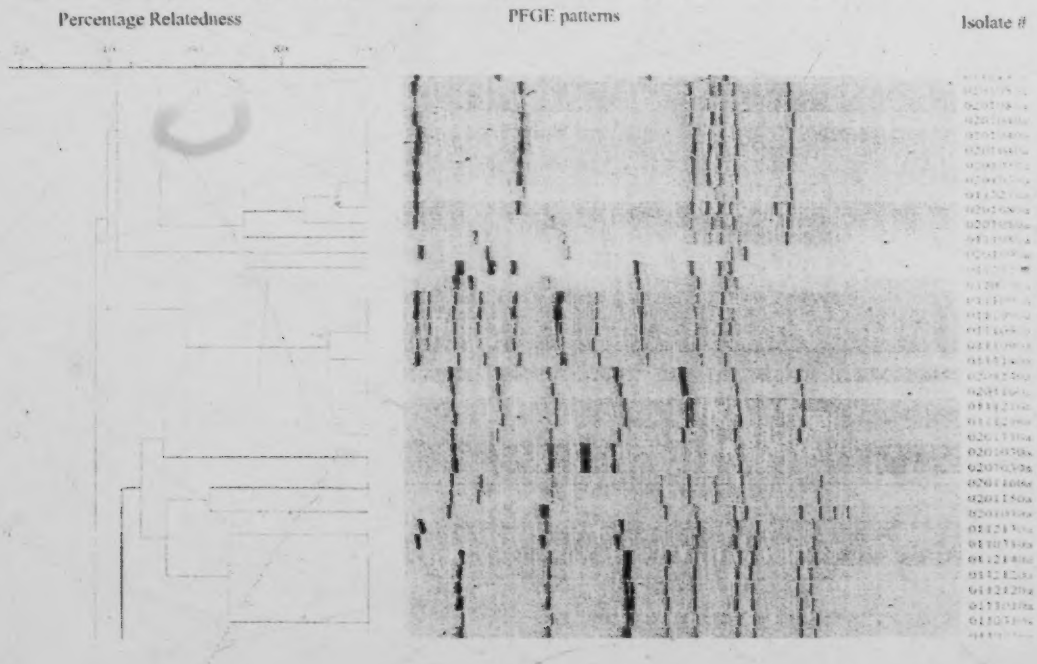


Figure 6-8 Example of pulsed-field gel electrophoresis dendrogram of *C. jejuni* and *C. coli* isolates illustrating the percentage relatedness, dendrogram tree and gel patterns analyzed



The *C. jejuni* isolates were grouped into 41 categories that were over 75% related, with a range of one to 67 isolates per group (mean of 8.73 isolates, S.D. of 13.84). There were 34 categories with fewer than 10 isolates, encompassing 120 isolates. Seven categories that were considered frequent, with 10 or more isolates.

The distributions for isolates of *C. jejuni* from cecal and retail samples relative to the 41 PFGE categories showed many categories that did not contain any isolates and generated 65 cells with expected counts under five. The scarcity of isolates within each of the categories would require a Fisher's exact test to establish whether there were significant differences between cecal and retail populations with regard to which of these categories they were occupied.

To establish whether any PFGE categories showed a potentially significant relationship with cecal or retail origin of an isolate, they were used in a discriminant analysis with a

logistic regression model. They were treated as categorical variables compared with the final category, which consisted of one isolate, and the outcome variable was defined as whether the isolate was from a cecal or retail sample. None of the individual categories was significantly associated with cecal or retail origin.

The seven categories that contained more than 10 isolates each were evaluated for relatedness. These categories accounted for 238 of the 358 *C. jejuni* isolates that produced PFGE patterns. The remaining 120 *C. jejuni* isolates were removed from analysis, because they consisted of relatively infrequent isolates. The descriptive characteristics of the PFGE categories with over 10 isolates are presented in Table 6-17. When the categories were compared by Pearson's chi-square test and Fisher's exact test for differences in proportions, there were no significant differences detected between cecal and retail isolates for membership in these categories.

Table 6-17 Descriptive characteristics of seven PFGE categories that each contained more than 10 isolates

PFGE category	Number *C. jejuni* cecal isolates	Number *C. jejuni* retail isolates	Total Isolates
Category A	7	9	16
Category B	19	17	36
Category C	17	29	46
Category D	31	36	67
Category E	5	11	16
Category F	7	15	22
Category G	12	23	35
Mean	17.38	27.38	44.75
S.D.	12.78	22.77	34.85

None of these categories provided significant distinction between the two groups of *C. jejuni* isolates when they were considered as categorical variables within a logistic regression model, with cecal or retail origin of the *C. jejuni* isolate as the outcome. When the combined category, containing all the remaining infrequent PFGE types, was

included in the logistic regression as a single category compared to these ten more common categories, there was no difference in ability of the model to discriminate cecal from retail isolates.

6.4.2 Results of the Percent Relatedness of PFGE Patterns

The percentage relationship between PFGE patterns and the patterns for the reference strains showed that the *C. lari* were so different from all the *C. jejuni* that the percentage relationship was very low and did not vary sufficiently among the *C. jejuni* isolates to be effective as reference strains so they were not analyzed further. The *C. coli* and *C. jejuni* reference strains were examined for correlation by Spearman's test which detected significant correlation among almost all of the reference strains. The coefficients are summarized in Table 6-18. Two strains of *C. coli*, v345 and v431 were the most unique, each strongly correlated with only two other strains. Strain v701 was significantly (at 0.01 level) correlated with six of the seven other chosen reference strains. This shared relatedness might cancel its effectiveness as a reference strain, or might indicate that it is the most balanced and representative of them all.

Table 6-18 Spearman's rho correlation coefficients for the selected reference strains of *C. coli* and *C. jejuni* (n = 358)

Reference Isolate	*C. jejuni* V535	*C. jejuni* V52	*C. jejuni* V54	*C. jejuni* V683	*C. coli* V345	*C. coli* V431	*C. coli* V729	*C. coli* V701
***C. jejuni* V535**	1.00							
***C. jejuni* V52**	-.263*	1.00						
***C. jejuni* V54**	-0.036	0.154*	1.00					
***C. jejuni* V683**	.321*	-0.152*	-0.066	1.00				
***C. coli* V345**	-0.052	0.000	-0.064	-0.023	1.00			
***C. coli* V431**	-0.083	-0.012	-0.155*	0.012	0.640*	1.00		
***C. coli* V729**	-0.541*	0.180*	0.378*	-0.290*	-0.021	-0.054	1.00	
***C. coli* V701**	-0.215*	0.294*	0.274*	-0.345*	.238*	0.064	0.326*	1.00

*** significant at 0.01 level**

The relationship between cecal and retail source of *C. jejuni* and the PFGE information was examined by comparing the relatedness values to each of the PFGE reference strains. Box-plots of these percentage relatedness variables showed comparable spread and limited skew when these variables were categorized by cecal or retail source of the *C. jejuni* isolate. The samples size was 139 cecal isolates and 219 retail isolates, which is quite comparable and samples were independent with very similar standard deviations, justifying use of the t-test to compare mean percentage relatedness for these variables between cecal and retail isolates.

The percentage relatedness of all *C. jejuni* isolates to the four *C. jejuni* reference strains did not show significant association with cecal or retail designation. The percentage relatedness to one *C. coli* reference strain (v701) was significantly associated with cecal or retail origin of *C. jejuni* isolates, and of interest because it was the strain that correlated with most of the other reference strains. This reference strain had a mean percentage relatedness to cecal isolates of 23.57% and mean relatedness to retail isolates of 21.14%. The mean difference of 2.43% was determined to be significantly different (95% CI: 0.15, 4.7; $p = 0.036$) indicating that this reference strain of *C. coli* was more closely related to strains of *C. jejuni* isolated from cecal samples than to those isolated from retail samples. There were no other significant differences for the relatedness to reference strains.

As an additional measure of relatedness, the mean of the percentage relatedness across all the reference strain variables was compared and did not differ between cecal and retail populations of *C. jejuni* by the T-test. The equal variance assumption was valid (Leverne's Test $p = 0.729$), but there was no difference in overall mean (mean difference = 0.71; 95% CI: -0.18, 1.61; $p = 0.115$).

The discriminant analysis with a logistic regression model was established to complete the evaluation of potential differences between cecal and retail isolates of *C. jejuni*. It

included variables that had been demonstrated to be associated with cecal or retail status of the isolate ($p < 0.25$), as well as variables that would be expected to be biologically significant. The initial model included log transformed MIC (log MIC) for erythromycin (significant), log MIC for each of the three fluoroquinolones (to control for confounding), % relatedness to *C. coli* reference strain v701 (significant) and percentage relatedness to the remaining reference strains (to determine if any might be important when considered with other factors).

The log MIC for erythromycin resistance and the percentage relatedness to *C. coli* reference strain V701 remained as the variables in the final model. The Hosmer-Lemeshow statistic was non-significant ($p = 0.11$) indicating that the model was an acceptable fit for the data. The model explained a relatively small proportion of the difference between *C. jejuni* isolates obtained from cecal and retail sources, but is still an indication that these isolates do differ. The log MIC for erythromycin was the strongest predictor of cecal or retail origin in the final model. The model estimates that for every unit increase in a *C. jejuni* isolate's log MIC to erythromycin, the odds ratio that the isolate was from a retail source compared with a cecal source was increased by 12.88 (95% CI: 4.11, 40.41; $p< 0.0001$). This is consistent with the univariate analysis that showed retail isolates of *C. jejuni* tended to be more resistant to erythromycin than isolates obtained from the cecal samples.

The logistic regression model estimated that the odds of a *C. jejuni* isolate being of retail origin compared with cecal origin decrease by 0.97 (95% CI: 0.95, 0.99; $p = 0.012$) for each unit increase in the *C. jejuni* isolate's percentage relatedness to reference strain *C. coli* V701. Therefore, the cecal isolates of *C. jejuni* are more similar to the *C. coli* reference strain than the isolates from retail samples.

CHAPTER 7

Discussion

7.1 Study A: Isolation of *Campylobacter* spp. from Ceca of Chickens

The objective of this study was to obtain an estimate of the population prevalence of *C. jejuni* in the ceca of Alberta broiler chickens arriving for slaughter. The sample of 400 cecal samples provided that 95% of random samples of this size, taken from the population, would provide a prevalence estimate that was within 5% of the true population prevalence. The unit of observation was the 6-week-old broiler population in Alberta during one production cycle (8 weeks) in the early spring of 2001, chosen so that every broiler chicken flock in Alberta would be shipped for slaughter during that time period.

7.1.1 Prevalence of *C. jejuni* in Cecal Samples

The prevalence of *Campylobacter* spp. detected in the ceca of broilers at slaughter was 39.25%, with 35% prevalence of *C. jejuni*, which was lower than isolation rates reported from intestinal sampling at slaughter in Canada (Prescott and Gellner 1984b) where 258/260 colonic swabs from 28/60 flocks were positive for *C. jejuni*. There are few reports of the prevalence of *Campylobacter* in intestinal samples in slaughter age birds reflecting regional differences. Most studies of intestinal sampling report prevalence within flocks in relationship to risk factors. One British study reported a crude prevalence at slaughter of 81.6% in broilers that represented 50% of the chicken production (Evans and Sayers 2000). Within-flock and between-flock prevalence were not estimated in our sample and direct comparison to these values in the literature is not possible. Isolation frequency from intestinal samples should not be compared with that from whole carcass sampling at slaughter because increased contamination is likely during processing (Oosterom *et al* 1983).

The lower prevalence of *Campylobacter* and *C. jejuni* obtained in this survey might suggest that isolation methods were less sensitive than those used by other researchers, or that *Campylobacter* survival was diminished by delays in sample handling. The culture of whole cecum rather than swabs would protect the *Campylobacter* organisms within their preferred, moist, micro-aerophilic environment in the cecal crypts during transportation to the laboratory. The tissue layer would provide a buffer to temperature and other environmental extremes that would reduce viability.

Culturing was initiated within 24 hours of sample collection to support optimal recovery. This was substantiated by no difference in isolation frequency between samples collected in Edmonton and those collected from the regional slaughter facilities.

Both direct and enrichment processes were used to enhance recovery of *Campylobacter*, under stringent quality control guidelines. Enrichment was observed to be less sensitive than direct culture in detecting positive samples and this step was eliminated after 100 samples. This was consistent with what has been reported and reasonably ascribed to enhanced competition from other organisms in the cecum that are also supported by enrichment culture, which is not selective (does not favor *Campylobacter*) (Musgrove *et al* 2001).

The low prevalence is most likely attributable to a seasonal effect, because sampling in this case was done during the late winter when prevalence in other surveys has been found to be lower (Thompson *et al* 1986, Wallace *et al* 1997, Willis and Murray 1997). A parallel cross-sectional study would be desirable to confirm or refute a seasonal effect within a large population and independent of farm or flock.

The odds of detecting *C. jejuni* did not differ among the four slaughter facilities nor did they differ when the four slaughter facilities were combined into the two slaughter

companies that they represented (Table 6-2). There was also no detectable difference among the three cities where the slaughter facilities were located. This was somewhat surprising because poultry diseases vary geographically through the province and the chicken populations that these facilities slaughter might be expected to differ. There is also integration between the slaughter company and the broiler chicken farmers. Chicken farming practices might depend on the associated slaughter company because slaughter company representatives often give the farmers production advice.

There was an effect on isolation of *C. jejuni* associated with weekday of sampling. The odds of a positive culture were higher for samples collected on Tuesdays relative to both Monday (OR: 1.91) and Thursday (OR: 2.85). Samples collected on Wednesday also had higher odds of positive culture than those collected on Thursdays (OR: 2.02).

The distribution of samples among weekdays was uneven and associated with city of origin because the regional facilities could not be sampled on Fridays and because both Edmonton facilities were large and had a greater sample allocation. Transportation stress and delay in culture were expected to reduce the isolation of *Campylobacter*, but the opposite effect was found, with samples collected on Thursday in Lethbridge having 6.38 times the odds of a positive culture as those collected in Edmonton on Thursday. Samples from Calgary and Edmonton on Thursdays were virtually identical (OR: 1.11). While this might suggest that the Lethbridge facility had a higher overall prevalence, the opposite effect was seen among samples taken on Tuesday. Lethbridge samples were less likely to be positive that those from Edmonton on that day (OR: 0.3) while samples originating in Calgary on Tuesday were more likely to be positive than Edmonton samples (OR: 3.75).

Weekday of sample collection was included in a multivariate model with slaughter city and slaughter facility. Neither of the latter had a significant effect on the odds of a positive culture, but the weekday remained a significant variable (Table 6-4). Therefore

when the city of origin and the slaughter facility (hence slaughter company), which were potentially important and associated with the weekday of collection, are controlled for, the day of the week that samples were collected still influenced whether *C. jejuni* was isolated, with both Tuesday (OR: 2.8) and Wednesday (OR: 1.9) having higher odds of positive culture than Thursday.

There was no biologically plausible explanation for the lower odds of a positive culture of *C. jejuni* from cecal samples on Thursday because this finding would require that factors related to the samples or the laboratory process were consistently less likely to produce a positive culture on that day of the week. It is unlikely that flocks slaughtered on Thursdays through this collection period would have a lower prevalence of *C. jejuni* because there were no links among them. Each flock was sampled only once in the time period, so repeat sampling from highly shedding flocks on Tuesday would not account for the finding. Samples were collected by the same person in each of the regions on every day of the sampling period and rare substitutions were not consistently on a given weekday. The transport containers were re-used, but were disinfected between uses and they were not re-used on a regular, consistent basis. No other factors could be consistently associated with the weekday of collection to account for the statistical association.

Laboratory factors that might explain the association of *C. jejuni* culture with weekday of sample collection included weekly changes in staff or procedures, but, as with sample collection, the same individuals were responsible for laboratory tasks through the entire study period. Media preparation did not differ and culture methods were consistent. The possibility of increasing contamination of the collection material or transportation containers was considered, but if this had been the case, samples collected later in the week would be expected to have higher isolation rates, and the opposite effect was found. The effect would also be expected to differ between regional facilities that had transportation delays and Edmonton samples that were processed on

the same day, but the regional effect was eliminated and the weekday effect remained. The majority of samples were obtained on Tuesday and Thursday, but there was no difference in sampling frequency between the two weekdays that might shift the association. The statistically significant result may simply be a spurious finding that is of no biological importance, but is important to consider in planning future studies since it is not possible to eliminate an effect associated with how chickens were transported to the slaughter facilities.

7.2 Study B: Isolation of *Campylobacter* spp. from Chicken Purchased in Retail Stores

The sampling of chicken purchased in retail stores was designed to reflect a prevalence of *C. jejuni* on whole fresh, never-frozen broiler chickens available for retail purchase at major grocery chains in the two urban centers in Alberta. This would represent the chicken available for home preparation to the majority of consumers in Alberta, because 52% of the people in the province reside in these cities (Alberta Municipal Affairs 2003). A total of 66% of Albertans live in the 15 cities in the province and an unknown additional percentage do much of their shopping in cities. There may be differences in the chickens available for purchase in smaller towns and villages, but a small sample from comparable groceries in Lethbridge did not differ from the overall results.

The distribution of retail store companies in this study reflected what stores were present in the cities where sampling was done and stores were chosen randomly to ensure that they were representative of the retailers present in these centers. There were numerous retail companies and retail brands and some were quite infrequent in the sample. This resulted in limited power to detect associations and some determinants were too uncommon to examine. Some relationships were identified during exploratory evaluation of data, but failure to detect an association was not conclusive evidence that there was none, due to lack of power.

Retail chickens were selected randomly to prevent staff from favoring larger, hence older, chickens that might be more likely to be positive for *C. jejuni* (Berndtson *et al* 1996b, Berndtson *et al* 1996a). There were 19 chickens within the sample that exceeded the 2 kg weight typical of broilers, placing them in the roaster category. These larger chickens were associated with specific stores (53% from retail company A and 37% from retail company B) and brands (68% were Other brand). There was no difference in the prevalence of *C. jejuni* between these larger chickens and the chickens under 2 kg and they did represent what was presented in retail stores for consumer purchase and so it was legitimate that they were retained as part of the study.

There was an uneven distribution of retail samples by weekday, associated with city of origin, reflecting sampling design and similar to that found with the cecal samples. The potential for this to influence culture results due to time and handling prior to culture was considered in the analysis of *C. jejuni* isolation results.

7.2.1 Prevalence of *C. jejuni* in Retail Carcass Rinses

The prevalence of *C. jejuni* in the carcass rinse samples was 53.2%. *C. lari* was isolated from 8 carcasses and this organism had not been grown from any of the cecal samples. There were small numbers of *C. coli* isolated (5% of total samples positive). The overall prevalence of *Campylobacter* was relatively low compared with what has been reported from other surveys of whole processed chicken. A previous Canadian study of whole carcass washes in Manitoba found *Campylobacter* in 84% of inspection passed carcasses (Powell *et al* 1995). Similar levels of 83% from whole body rinse of broilers in England (Jorgensen *et al* 2002), 94% from retail chicken in Ireland (Moore *et al* 2002), and 82% from retail chicken (Dickins *et al* 2002) have been reported. The results of this study are closer to the survey results of 44% reported for three states surveyed in the USA (Rossiter *et al* 2001a). The relatively low prevalence may reflect the fact that sampling in this survey was done during April and May, because late summer and autumn are the seasons most associated with high prevalence.

The prevalence of *C. jejuni* in retail chicken was higher than the prevalence among chickens arriving for slaughter. An increase in prevalence has been documented within a group of chickens between the time of slaughter and when they leave the slaughter facility (Oosterom *et al* 1983). This was attributed to cross contamination and spread of the organism in the chilling tanks as well as within the atmosphere and on the hands of workers in the slaughter facility. *Campylobacter* survives very well in a damp environment and can be protected by biofilms that are present on surfaces in slaughter and processing facilities (Trachoo *et al* 2002). The organism may also be retained in the feather follicles of the skin, which represents an atmosphere with limited oxygen that the bacteria prefer. The time delay between slaughter and sale, with the chicken held in a moist environment and in a sealed bag, that favors microaerophilic conditions, could also increase the bacterial load on each carcass if temperatures rose to a level that would favor replication. *Campylobacter* grows best at 42°C, but some growth would take place at lower temperatures and resulting higher bacterial loads would be more readily detected by culture.

Contamination of carcasses may also occur from other sources. While humans do not shed the bacteria for extended periods of time (Endtz *et al* 1991), intermittent shedding may occur. It is also possible that cross-contamination occurs through organisms in wet areas of the retail or packing environment. The bacteria are also commonly shed by other species and cross contamination from pork or other meats might occur. The isolation of markedly different strains of *C. jejuni* from the cecum of broilers and whole retail carcasses would suggest that there are alternate sources of contamination or that the bacteria change during their replication on the carcass.

The prevalence of *C. jejuni* in this survey did not differ among the three cities, indicating that despite the differences among the cities for retail stores and brands, the overall odds of a retail chicken being positive for *C. jejuni* are similar regardless of

which city in Alberta it was purchased in (Table 6-6). One retail store company did have substantially lower odds (OR: 0.35) of chicken being positive for *C. jejuni* relative to the other retail stores. The brand A that was sold exclusively by this store had lower odds of being positive for *C. jejuni* than any of the other retail brands had. The group of less common brands (Other) also had lower odds of a positive culture than any of the common brands, except for Brand A had.

One factor that could account for lower odds of *C. jejuni* isolation for selected brands or retailers is that they purchased chickens consistently from a less contaminated source. The prevalence did not differ among slaughter facilities in this study based on chickens arriving for slaughter but this does not eliminate the possibility that contamination occurred during processing resulting in some slaughter facilities producing a more heavily contaminated final product. Some retailers may also store chickens at more consistently low temperatures that prevent the growth of *C. jejuni*. Excessive chilling or freezing may have been an influence in lowering *Campylobacter* levels.

Campylobacter are quite sensitive to freezing injury and a 17% lower prevalence was reported in comparing previously frozen chicken to fresh (Moore *et al* 2002). While retail staff persons were questioned to ensure the chickens purchased were fresh and never frozen, it was possible that the staff from some retailers consistently responded incorrectly or that previously frozen chickens, or partially frozen chickens were being sold as fresh. This final factor could account for the lower prevalence in Brand A from Retailer E, but was less likely to account for the lower prevalence among the three brands in the Other category.

Retail store company and retail brand were examined in a logistic regression model that included the weekday of sampling and store city to control for them as potential confounders of the effect on isolation of *C. jejuni*. Retail brand was a significant predictor ($p = 0.035$) of whether *C. jejuni* would be isolated. All the common brands

had substantially higher odds (Table 6-7) of being positive for *C. jejuni* than Brand A or the combined brand Other. The confidence intervals were very wide, an indication of the uncertainty around the estimates of the odds ratios that reflects the relatively small sample sizes in each category. This did confirm that there were brand related differences in the odds detecting *C. jejuni* in retail chicken. This might be accounted for by different slaughter facility sources of the chickens or by differences in how the individual brands are processed. The major poultry processors have both standard and premium products related to nutrition of the chickens during the growing period and factors like whether they are chilled in water or by cold air. These factors were not evaluated in this study and may account for the brand related differences in *C. jejuni* prevalence. These findings suggest that further studies evaluating the source and handling of poultry between the slaughter facility and the retail store may clarify the contributing factors to *C. jejuni* colonization of whole retail chicken.

7.3 Antimicrobial Resistance of *Campylobacter* spp. from Cecal and Retail Samples

Fluoroquinolone antibiotics are derivatives of nalidixic acid with a common mechanism of action and typically bacteria are cross-resistant (Endtz *et al* 1991, Reina *et al* 1992, Taylor and Courvalin 1988b). This was substantiated among both cecal and retail isolates in this study. All *C. lari* isolates were from retail chickens and were resistant to all three fluoroquinolone antibiotics. Resistance to nalidixic acid is one species characteristic that distinguishes *C. lari* from *C. jejuni* and *C. coli*.

There were 17 *C. coli* isolates from cecal samples and 35% were resistant to all the fluoroquinolone antibiotics. Of the 21 *C. coli* isolates from retail chickens, only one was resistant to enrofloxacin, but sensitive to the other fluoroquinolone antibiotics. The *C. coli* isolates from the cecal and retail sources differed markedly in sensitivity to fluoroquinolones, but investigation of *C. coli* and *C. lari* was not the focus of this research and will be explored further in a future publication.

7.3.1 Antimicrobial Resistance of *C. jejuni* Associated with Slaughter Variables

The *C. jejuni* isolates from company X were significantly more resistant to ciprofloxacin than those from company Y, but there was only a borderline difference with respect to enrofloxacin, which has been used in the poultry industry (Table 6-14). The slaughter companies frequently have a veterinarian on staff to provide advice to the poultry farmers that sell their chickens to that slaughter company. This would be expected to result in some differences in treatment regimes between the two companies. Enrofloxacin has been available and prescribed for use in poultry, but ciprofloxacin has not. Use of enrofloxacin during the life of the flock would be expected to result in an increase in resistance at the time the drug was used in the birds (Jacobs-Reitsma *et al* 1994b, Wretlind *et al* 1992a). Perhaps the components of resistance that are common between enrofloxacin and ciprofloxacin are retained in the intestinal population of *C. jejuni*, while the components of resistance to enrofloxacin need more immediate selection pressure to be expressed. The use of enrofloxacin in commercial broilers tends to occur at approximately 3-4 weeks of age when it would be used for control of *E. coli* septicemia. Although resistance to fluoroquinolones has been retained in the absence of selection pressure (Wretlind *et al* 1992a), perhaps not all components of that resistance are retained well in *C. jejuni* until broiler chickens are 6 weeks of age.

The differences in ciprofloxacin and enrofloxacin resistance between the two slaughter companies originated from a difference between the two large facilities, both located in Edmonton as well as a difference between one large facility (A large) and one of the regional facilities (C small). The difference between facilities in the same location indicates that it is not attributable to geographic location. Factors such as poultry feed supply companies, water quality and supply, access to laboratory facilities for diagnosis and advice, as well as climate and altitude differ considerably between the Edmonton area and the areas around Calgary and Lethbridge. These have an impact on how broilers are managed and what they are exposed to during life as well as the frequency of diseases. While these factors might not be expected to have a direct influence on

whether the *C. jejuni* flora from chickens arriving at these facilities was resistant to ciprofloxacin, it was possible that this resistance was genetically linked to factors that favor *C. jejuni* survival in the intestine, and that selection for resistance to ciprofloxacin in *C. jejuni* might be secondary. The fact that the differences in resistance are present in separate areas of the province suggests that these environmental influences are not important in this case.

The borderline difference in *C. jejuni* resistance to erythromycin between the two slaughter companies became significant when individual slaughter facilities were compared (Table 6-15). Although erythromycin was not commonly used in commercial poultry, chickens arriving for slaughter at the D small slaughter facility may have been more likely to be exposed to an antibiotic that would induce cross-resistance to erythromycin. The poultry producers in the vicinity of the two smaller slaughter facilities that are located far from the poultry diagnostic laboratory, located in Edmonton, and may be more likely to make decisions on medication of their flocks without consulting a poultry veterinarian. This could result in treatment with drugs like tylosin during the life of the flock. *C. jejuni* isolates that are resistant to erythromycin are consistently resistant to these drugs (Taylor and Courvalin 1988a) so cross resistance would result.

7.3.2 Antimicrobial Resistance of *C. jejuni* Associated with Retail Variables

There was a significantly lower mean rank of the MIC to nalidixic acid among *C. jejuni* isolates from Edmonton relative to those from Calgary retailers. The fact that a difference was detectable for only one antibiotic, without any cross-resistance to the other fluoroquinolones suggests that this was not a biologically significant finding, although the number of isolates examined allowed detection of a statistically significant finding.

The comparisons between brands were only carried out for the three most frequent brands, Brand B, C and D. Brand D was a source of *C. jejuni* isolates that were somewhat less resistant to the enrofloxacin and ciprofloxacin than Brand B based on categorical and MIC comparisons. They were less resistant than Brand C based on the actual MIC values but not through the comparison of categorical resistance. A brand association with resistance to antibiotics could occur if the brand were one that was integrated with a slaughter company that influenced production practices on the broiler farms. Brand D was one of two brands in this study that were linked with a major slaughter company. This could explain the difference in resistance of the *C. jejuni* isolates from this brand, if use of fluoroquinolone antibiotics was less frequent on farms that supplied the corresponding slaughter company. The link between the brand and the slaughter company was not explored further because including additional detail would breech confidentiality and could represent a threat to the business of the major slaughter companies that would be based on speculation.

There was an association with resistance of *C. jejuni* isolates from two retail stores with lower resistance to several antibiotics. Stores A and E tended to yield isolates of *C. jejuni* that were more sensitive to the fluoroquinolones and erythromycin than retail stores B, C or D (Table 6-16). Retail stores and retail brands were related and while all retail stores sold more than one brand of chicken, some brands were offered only by one retail company. The brand effect can be more closely related to the source of contamination and both store A and store E sold a high percentage of Brand D (Fig 6-3), which was the brand that produced isolates of *C. jejuni* that were more sensitive to fluoroquinolone antibiotics. This indicated that the retail store effect was likely a reflection of the brand effect and because the brand was more closely linked to production and processing, it would be more accurate.

7.3.3 Comparison of Antimicrobial Resistance Patterns Between *C. jejuni* from Cecal and Retail Sources

Approximately 12% of the total of 362 *C. jejuni* isolates were resistant to enrofloxacin and there was no difference in the odds ratio for resistance between cecal and retail isolates (Table 6-10). The odds of resistance also did not differ between any pairs of the fluoroquinolone antibiotics within either the cecal or retail isolates of *C. jejuni*. While the median MIC values were slightly higher for retail isolates, the ranges, interquartile ranges, minimum and maximum MIC values were virtually identical between the cecal and retail isolates (Table 6-11). The association among the isolates for resistance to fluoroquinolone antibiotics was substantiated by the high correlation coefficients among the MIC and log MIC values (Tables 6-12, 6-13). This suggests that the level of resistance of *C. jejuni* isolates to the fluoroquinolone antibiotics would not distinguish the cecal and retail populations.

There was only a weak correlation between resistance to any of the fluoroquinolone antibiotics and erythromycin, as would be expected because the mechanism of resistance differs (Taylor and Courvalin 1988a, Taylor 1992, Tenover *et al* 1992). The majority of *C. jejuni* isolates from both cecal and retail specimens were of intermediate sensitivity to erythromycin, but *C. jejuni* isolates from cecal samples had a mean MIC that was 1.12 units below the retail isolates; the retail isolates were significantly more resistant to erythromycin. This substantiates a difference between these two populations of *C. jejuni* and may suggest that the *C. jejuni* that are present on retail chicken arise from sources in addition to those carried in the ceca of chickens arriving for slaughter.

Cecal and retail isolates differed more with respect to erythromycin resistance than sensitivity to fluoroquinolones. The slaughter chicken variables were more strongly associated with fluoroquinolone resistance of *C. jejuni* isolates than with the erythromycin resistance. This suggests that the variance in erythromycin resistance

arose from differences between the cecal and retail isolates of *C. jejuni* and is less associated with factors related to rearing broiler chickens. The difference between the cecal and retail isolates may be attributable to the time frame in which the cross-sectional studies were done, the slight shift in season between these surveys or it may relate to *C. jejuni* on retail carcasses arising from more sources than contamination from intestinal content of chickens at slaughter.

7.4 Pulsed-Field Gel Electrophoresis Patterns

The PFGE patterns that were produced for 358 of the *C. jejuni* isolates were very diverse and were similar to those reported from previous studies in terms of the number and thickness of bands (Steele *et al* 1998, Thomas *et al* 1997). The band patterns were readily distinguished and there were 41 categories that contained strains with greater than 75% of their band pattern in common, which was sufficient to consider them closely related. Only seven categories were frequent, with more than 10 isolates per category. The value in examining the PFGE patterns was to determine whether cecal and retail source isolates of *C. jejuni* from these samples could be discriminated based on the patterns expressed by members of the populations.

Membership of a *C. jejuni* isolate in one of the 41 related categories was not associated with whether the isolate was of cecal or retail origin. The low numbers of isolates for this comparison reduced confidence in this conclusion, and comparison of the 10 most frequent categories revealed no differences in the proportion of cecal or retail isolates in these groups either. Neither categorical relatedness variable provided discrimination when included in a logistic regression model.

An analysis of the categories that were most frequent would 'address the means but ignore the extremes' and in this case, the extreme isolates might be those that would permit discrimination. In order to capture the range of pattern variation among the PFGE types, reference strains of *C. coli* and *C. jejuni* were used for comparisons. The

percentage similarity provided a unique variable and multiple reference strains would more accurately pinpoint each isolate, provided there was not excessive co-linearity among the reference strains.

The four strains of *C. coli* and *C. jejuni* that were distinct from the majority of strains and yet sufficiently similar to serve as reference strains were evaluated by a t-test and one reference strain was significantly associated with cecal or retail origin of the *C. jejuni* isolates, showing a mean difference of 2.43% relatedness between cecal and retail isolates. This reference *C. coli* strain (v701) was more closely related to cecal than the retail strains of *C. jejuni*. The significance of this relationship to discriminate between cecal and retail isolates in these samples was confirmed by the logistic regression, in which these variable were controlled for the log MIC of the fluoroquinolone antibiotics and erythromycin. The percentage relatedness to *C. coli* reference strain and the log MIC of each isolate to erythromycin were the two distinguishing factors between isolates of cecal and retail origin.

The percentage relatedness to a reference strain of *C. coli* did not have a practical application, nor is there a foundation in biological science for using this as a means to differentiate the sources of isolates. This was examined as a means to determine whether it was possible to document differences between two *C. jejuni* populations in a way that would compare their similarities and differences. This is difficult to do with organisms that have a small number of bands. The fact that 7 of 8 of the possible reference strains did not distinguish between the source of the isolates suggests that the capacity of one strain to differentiate them might not be biologically important. The distinction may be spurious. The fact that the cecal and retail isolates do differ with respect to this reference strain, suggests that differences can be quantified in this way, for comparison purposes. It may be possible to refine the method or the choice of reference strains to adapt it practically. The results do support that there are predictable

differences between the populations of *C. jejuni* that were isolated from cecal samples of broilers at slaughter and those isolated from whole body washes of retail chickens.

7.5 Limitations of the Research

There were multiple factors that restricted the validity and generalizibility of this research. True random sampling of slaughter facilities and retail stores was not logistically feasible although attempts were made to randomize when possible. There may have been unknown biases present. It was possible that submissions from individual farms were associated with the days or times that slaughter facilities were sampled. The sampling was spread throughout a full production cycle because every broiler barn in Alberta would have been in production and shipped birds to slaughter during that time period. There was no reason to expect that any individual farm or farmer would have a greater chance of having birds from his flocks sampled, except because his production contributed proportionally more to the provincial total than another farmer. This would be desirable from the perspective of obtaining a provincial prevalence estimate.

There may have been a systematic difference in how ceca or carcasses were collected or handled from one source that would be associated with reduced or enhanced culture potential. Some bias was possible because visits to retail stores were made on a convenience basis in order to sample the highest number of stores possible with the least travel time and cost. Typically the retailers in the general vicinity of each other represent competitive companies and it was not likely that they would have a common source of chickens or be likely to have deliveries from the same source on the same day as they were sampled.

The total sample sizes for both samples were established for an estimate of overall prevalence and not to permit comparison of isolates with risk factors from the sources. The power to draw conclusions from these analyses was limited. The comparisons were

explored, and when positive associations were present, they could be documented. Failure to demonstrate a difference does not confirm that the populations were the same because of the relative lack of statistical power inherent in exploring data beyond the initial research questions.

It was not possible to sample chickens through the entire production chain. If ceca could have been collected at slaughter, and the carcass cultured when it left the slaughter facility, and then after transportation to the retailer, and at time of sale, the associations between *C. jejuni* from cecal content and those on retail carcasses would be much clearer. It was not feasible to sample slaughter chickens and those purchased at retail stores concurrently because of the maximum sample size that could be cultured by the laboratory. While either of these methods would have allowed stronger associations to be drawn about how and whether *C. jejuni* isolates differ between those in the cecum of chickens at slaughter and those on retail chicken, this initial survey to establish baselines for the two categories of isolates was a practical first step.

Although the majority of fresh chicken sold by Alberta retailers is produced in the province, it was not possible to be certain that the retail carcasses originated in the slaughter facilities that were sampled in Study A. While direct associations between the isolates from the two populations cannot be made, comparisons are valuable because livestock producers in a province are typically held accountable in public opinion for problems that occur in the retail product. There is also a degree of integration between poultry producers, the companies that own the slaughter facilities and the brands that are marketed in Alberta. While direct comparisons between isolation rates and characteristics of *C. jejuni* from the slaughter facilities owned by a company and the brand that it markets are not supported by this research design, a more general comparison between the isolates of cecal and retail origin was done.

CHAPTER 8

Conclusions and Recommendations

The crude prevalence of *C. jejuni* in broiler chickens in Alberta was estimated to be 35% in cecal content of chickens at slaughter and 53% in whole body wash of chickens available for retail purchase. These estimates were based on samples taken during consecutive 8-week time periods that correspond to a full production cycle of Alberta broiler chickens. Approximately 12.5% of *C. jejuni* isolates in both populations were resistant to enrofloxacin. The prevalence and the level of resistance to enrofloxacin and other fluoroquinolone antibiotics were lower than reported in the literature, perhaps due to the season in which sampling was done. Additional prevalence surveys are desirable on an ongoing basis to establish whether a seasonal effect is present, and a sampling during the summer months might increase the number of positive outcomes to provide greater power in evaluating some associations as well. Monitoring is also important on an ongoing basis in Alberta because poultry production quality assurance programs are being implemented on farms, and the prevalence of *C. jejuni* in chickens arriving at slaughter might be a potential indicator of the effectiveness of these programs.

The isolation of *C. jejuni* from ceca of chickens at slaughter was associated with the day of the week in which the samples were collected. This association could not be explained based on possible biases or biological plausibility and may be a spurious result. The weekday of collection was not a variable that this research was designed primarily to evaluate, but was an association that appeared when this variable was being controlled for in other comparisons. In order to determine whether there are factors associated with weekday of slaughter, a cross-sectional study might be designed to specifically address the question of whether the weekday of sampling has an impact on the prevalence of *C. jejuni* when variables of sample collection and processing are strictly controlled.

The isolation of *C. jejuni* from retail samples was associated with retail brand as well as marginally with the day of the week samples were collected. The brand association applied to the resistance of these isolates to ciprofloxacin and erythromycin as well. Further research would be justified to explore the associations among integrated poultry slaughter facilities and retail brand with respect to whether prevalence of *C. jejuni* or the level of antibiotic resistance is impacted by processing methods, such as water or air chilling. It would also be desirable to investigate the relationship between antibiotic use during broiler chicken production and the resistance of *C. jejuni* isolates that are obtained from slaughter facilities that receive chickens from these farms. This type of study would require full cooperation of the processing facilities and the farms that supply them, because of the implications of research findings for their business practices, but it probably represents one of the most promising areas of future investigation.

The *C. jejuni* isolates from the two source populations could be distinguished with respect to their resistance to erythromycin and their PFGE patterns expressed as percentage relatedness to a reference strain of *C. coli*. The two source populations were not the same. Whether these differences were a result of random genetic variation, were related to differences in the time period in which the populations were sampled, or differed for some unknown reason, cannot be established. The fact that the two sets of isolates were different suggests that more research to trace isolates from slaughter to retail would help determine whether all the isolates of *C. jejuni* that are present on retail chicken arise from contamination with gut content of chickens. The potential to link this type of tracing to virulence factors may establish whether controlling *C. jejuni* in the intestine of poultry will have an impact on the occurrence of campylobacteriosis in humans, which is the ultimate concern with respect to investigation of these bacteria.

Future research should focus on determining the relationship of *C. jejuni* isolation with resistance to fluoroquinolone antibiotics as well as erythromycin through the slaughter process and to the retail shelves. Useful objectives would be determining whether the resistance is altered by processing methods or is dependent upon the farm source of the chickens and investigating the link between retail chicken brand and *C. jejuni* isolation through a study specifically designed to address these questions during the summer months when isolation rates would be higher.

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Appendix A

Sample Size calculation

An estimate of the population proportion is based on the sample proportion bounded by a precision interval (L). This interval depends on the degree of confidence required for the estimate (α%)) and the sample size (n). It is illustrated by the formula for a confidence interval (where CI is confidence interval and $\bar{X}$ is sample mean):

$$CI = p \pm Z_{\alpha/2}\sqrt{\frac{P(1-P)}{n}}$$

To determine the sample size (n), the precision interval:

$$L = Z_{\alpha/2}\sqrt{\frac{P(1-P)}{n}}$$

is solved for 'n':

$$n = \frac{Z_{\alpha/2}{}^{2} * p * (1-p)}{L^2}$$

The Zα/2 is set at 1.96 for a 95% confidence interval.

With binomials, the variance equals p * (1-p), where p is the proportion (prevalence). Thus, the variance will be maximal when p = 0.5, which will give the maximum sample size needed. The most reasonable estimate of prevalence is approximately 0.5 based on prior prevalence surveys of *Campylobacter* and since the vast majority of *Campylobacter* isolates from poultry samples are *C. jejuni*, the sample size estimate will not differ depending on whether all *Campylobacter* or *C. jejuni* specifically is the target. If the prevalence varies, either higher or lower than 0.5, the additional sample size will serve to increase precision.

Given these values and solving for n, we arrive at:

$$n = \frac{(1.96)^2 * (0.5)^2}{(.05)^2}$$

n = 368.64 or 370 samples

A random sample of 370 samples would provide an estimate of the prevalence of *Campylobacter* shedding in the Alberta broiler chicken population and similarly in the Alberta retail chicken population. There is a 95% probability that the true population prevalence will fall in a range 5% on either side of the sample estimate obtained by a random sample of 370 chickens. If the population prevalence is considerably different from 50%, the precision of the range, or the degree of confidence will be increased accordingly.

The total population size is a factor only when the sample size will exceed about 10% of the population size, since when the sample is proportionally large, the standard deviation tends to be over-estimated. The finite population correction (1- f, where f = sample fraction of population) can be applied for this, but is not required in this case, since the broiler population is 6 million.

Appendix B

Glossary of Terms

Chi-squared test – is a test generating a statistic that measures how well the observed data in a row by column contingency table fit the null hypothesis that their distribution is random, as it would be if the observations were independent (not associated).

Confidence interval (CI) – is the range of values within which the true magnitude of an association is captured with a specified degree of assurance. A 95% confidence interval is typically interpreted as a 95% chance that the population parameter will fall within the confidence interval. It is more precisely interpreted as 95% of all the confidence intervals that could be established from repeated samples of a population will contain the true population parameter.

Contingency table – is a tabular representation of the number of observations from a sample that are within the defined categories of the exposures and outcomes of interest. The most typical example (where a, b, c and d are counts) is:

	Outcome (yes)	Outcome (no)
Exposure (yes)	a	b
Exposure (no)	c	d

Fisher's Exact test – is a variant of the Chi-squared test that gives an accurate estimate of the association in a contingency table when the count in the cells of the table is less than 5.

Incidence – is the number of new occurrences of a condition that develop in a population of individuals who are vulnerable during a specific time interval.

Logistic regression – is a mathematical model that describes the association between multiple variables and a dichotomous outcome variable. It is based on the logistic function of the dichotomous variable and estimates the impact of each predictor variable on the odds of the outcome variable. It also provides an estimate of confidence intervals for these effects.

Mann-Whitney U-test – is a test that generates a statistic based on the sum of the ranks of individual observations and compares these rank sums between two groups of observations. It is a non-parametric test, so is a useful alternative to the T-test when the data violates the assumptions of the T-test.

Odds – is the ratio of the number of individuals within a group who are exposed to the number of individuals who are not exposed. The group is typically one that either is or is not affected by the condition of interest). This is (a/c) or (b/d) from the contingency table above.

Odds ratio (OR) – is the ratio of the odds of exposure in an affected group to the odds of exposure in an unaffected group. This is (a/c) / (b/d) which reduces to the formula ad/bc from the contingency table above.

Prevalence – the proportion of individuals in a population who are affected by a condition at a specific point or over a specified period in time

Relative risk (RR) – is the ratio of the incidence of disease in an exposed group to the incidence of disease in an unexposed group. This is (a/a+b) / (c/c+d) from the contingency table above.

T-test – is a test that generates as statistic to determine whether the mean of one group of observations differs from the mean of another group of observations.

Variable – is a characteristic can be observed and measured and can assume different values for individuals in a population. Variables may assume different numerical values, may represent different categories or may be a rank in an order along a scale.